Third Edition

Fundamental Food Microbiology

Bibek Ray



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Dedication

To my parents, Hem and Kiron, and my family

Preface to the Third Edition

In the third edition, substantial changes have been made in most of the chapters and in their logical arrangement. In addition, one new chapter has been added. The chapter on microbial stress has been written to include various manifestations of bacteria under stress and their importance in food microbiology. As before, this book is written primarily for students taking undergraduate food microbiology courses. However, it can be used as a reference in other related courses in many disciplines as well as by professionals engaged directly and indirectly in food-related areas. I thank Elizabeth Smith for her excellent typing and editing in the preparation of the manuscript. Finally, I thank my students for their helpful suggestions, especially for the new materials included in this edition.

Preface to the First Edition

Between the time I first studied food microbiology as an undergraduate student and now, the discipline has undergone a radical change. This change is well expressed by Dr. David Mossel of the Netherlands in his letter published in *ASM News* (59, 493, 1993): from "no challenge in plate count and coliform scouting" to "linkage of molecular biology to food safety (also food bioprocessing and food stability) strategies — proclaim a new era in food microbiology." This transition was necessary to meet the changes that occurred in the food industry, especially in the U.S. and other developed countries. The necessary knowledge, techniques, and expertise for this transition were available. This book reflects this transition from the traditional approach to an approach to meet the needs of those who are directly or indirectly interested in food microbiology.

Introductory food microbiology is a required course for undergraduates majoring in food science. In some form it is also taught in several other programs, such as microbiology, public health, nutrition and dietetics, and veterinary science. For the majority of food scientists, except those majoring in food microbiology, this single course forms the basis of the study of microorganisms and their interactions to food. Similarly, for the latter group, food microbiology is probably the only course that provides information on the interaction of food and microorganisms. This book was written with the major objective of relating interaction of microorganisms and food in relation to food bioprocessing, food spoilage, and foodborne diseases. Thus, it will be useful as a text in the introductory food microbiology courses taught under various programs and disciplines. In addition, it will be a valuable reference for those directly and indirectly involved in food and microbiology, including individuals in academic institutions; research institutions; federal, state, and local government agencies; food industries; food consultants; and even food lobbyists.

The subject matter is divided into seven sections. For undergraduate teaching, the first six sections can be taught as a semester course; Section VII (Appendices) can be used as advanced information for an undergraduate course which contains materials that are either taught in other courses, such as advanced food microbiology, or food safety courses and laboratory courses. Section I describes the history of food microbiology, characteristics of microorganisms important in foods, their sources, and significance. Section II deals with microbial growth and metabolism of food, and the significance of microbial sublethal injury and bacterial sporulation in foods. Section III explains the different beneficial uses of microorganisms, which include starter cultures, bioprocessing, biopreservation, and probiotics. Section IV deals with spoilage of foods by microorganisms and their enzymes and methods used to determine food spoilage. In addition, there is a chapter on problems and solutions of some emerging spoilage bacteria in refrigerated foods. Section V deals with foodborne pathogens associated with intoxication, infections, and toxicoinfections and those considered to be opportunistic pathogens, as well as pathogenic parasites and algae. In addition, a chapter has been included on emerging pathogens and a chapter on indicators of pathogens. Section VI discusses different methods used to control undesirable microorganisms for the safety and stability of food. A chapter on new

nonthermal methods and a chapter on the hurdle concept in food preservation are included.

The materials in each chapter are arranged in logical, systematic, and concise sequences. Tables, figures, and data have been used only when they are necessary for better understanding. At the end of each chapter, a limited list of selected references and suggested questions have been included. To reduce confusion, especially for those not familiar with the constant changes in microbial genera, three first letters have been used to identify the genus of a species. The index has been prepared carefully so that the materials in the text can be easily found.

I thank Mrs. Deb Rogers for her excellent performance in typing the manuscript. Finally, I thank my students, who, over a period of the last 20 years, have suggested what they would like to have in a food microbiology course. Their suggestions have been followed while writing this text.

Preface to the Second Edition

It is gratifying to find that CRC Press showed interest in a second edition within 3 years of the initial publication of *Fundamental Food Microbiology*. As indicated previously, this book was written primarily as a text for undergraduate food microbiology courses. The main objective was to provide basic and applied information in as many areas as possible in about 500 pages. In the second edition, the materials were carefully edited and new information included to keep it up to date. As before, the second edition will be important not only to undergraduate students in a food microbiology course, but also as a valuable reference book to graduate students in the food science area, to individuals associated with the science, application, production, and regulation of foods as related to microorganisms in academic institutions, research institutions, and food-testing laboratories. In addition, short course organizers, food consultants, food industries, food regulatory agencies, and food science professionals will find this book valuable to understand and solve problems associated with microbiological aspects of food. I thank Mrs. Deb Rogers for her excellent typing and editing in the preparation of the manuscript and the students in the food microbiology class for their helpful suggestions, including the new material in the second edition.

The Author

Bibek Ray, Ph.D., was a professor of food microbiology in the Department of Animal Science at the University of Wyoming, Laramie. Professor Ray earned B.S. and M.S. degrees in veterinary science from the University of Calcutta and University of Madras, in India, respectively. He received his Ph.D. in food science from the University of Minnesota in 1970 and joined the faculty in the Department of Food Science, North Carolina State University, and then the Department of Biology at Shaw University, both at Raleigh. He joined the University of Wyoming in 1981. There he expanded his research to intestinal beneficial bacteria, bacteriocins of Gram-positive bacteria, and high hydrostatic pressure preservation of food along with his previous research activities in the area of microbial sublethal injury. He also taught courses in food microbiology, food fermentation, food safety, and a course titled "Safety of Our Food" to nonscience undergraduates. His laboratory was involved in extensive and thorough studies in both basic and applied areas of the bacteriocin pediocin AcH from *Pediococcus acidilactici* H. In addition, his group studied various aspects of bacteriocins produced by *Lactococcus, Leuconostoc, Lactobacillus,* and *Pediococcus* as well as *Bacillus* and *Staphylococcus* spp. He received research funding from the National Science Foundation, American Public Health Association, National Live Stock and Meat Board, United States Department of Agriculture, United States Army Research, North Atlantic Treaty Organization (with Turkey) and Binational Agriculture Research Development Agency (with Israel), Wyoming Development Fund, and the industry. Before retirement, he was studying the combined effect of bacteriocins, ultrahigh hydrostatic pressure, and pulse field electricity and sublethal injury on the destruction of microbial cells and spores and its application in food preservation. In addition, Dr. Ray established collaborative research programs with research institutes and universities in Turkey, Israel, India, Indonesia, and France.

Professor Ray has published more than 100 research articles, reviews, book chapters, proceedings articles, and popular articles on food microbiology. He has also edited four books: *Injured Index and Pathogenic Bacteria* (1989) and *Food Biopreservatives of Microbial Origin* (1992, with Dr. M.A. Daeschel), both published by CRC Press, Boca Raton, Florida; *Lactic Acid Bacteria: Current Advances in Metabolism, Genetics, and Applications* (1996, with Dr. Faruk Bozoglu), Springer, New York; and *Novel Processing and Control Technologies in the Food Industry* (1999, with Dr. Faruk Bozoglu and Tibor Deak), IOS Press, Washington, D.C. He was a member of the American Society for Microbiology and the Institute of Food Technologists and a Fellow of the American Academy of Microbiology. He also served on the editorial boards of the *Journal of Food Protection*, *Applied and Environmental Microbiology*, and the *Indian Journal of Microbiology.* In 1994, Professor Ray was awarded the University of Wyoming Presidential Achievement Award in recognition of his excellence in academic performance. He retired from the University of Wyoming in September 2002. He is presently involved in developing a center to improve health, education, and economic conditions of underprivileged people in his village of birth in India and spends a great deal of his time in the village.

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SECTION I

Introduction to Microbes in Foods

Microorganisms are living entities of microscopic size and include bacteria, viruses, yeasts and molds (together designated as fungi), algae, and protozoa. For a long time, bacteria have been classified as procaryotes (cells without definite nuclei), and the fungi, algae, and protozoa as eucaryotes (cells with nuclei); viruses do not have regular cell structures and are classified separately. In the 1990s this classification changed, and will be briefly mentioned in Chapter 2. Microorganisms are present everywhere on Earth, including humans, animals, plants and other living creatures, soil, water, and atmosphere, and they can multiply everywhere except in the atmosphere. Together, their numbers far exceed all other living cells on this planet. They were the first living cells to inhabit the Earth more than 3 billion years ago and since then have played important roles, many of which are beneficial to other living systems.

Among the microorganisms, some molds, yeasts, bacteria, and viruses have both desirable and undesirable roles in our food. In this section, importance of microorganisms in food, predominant microorganisms associated with food, sources from which they get in the food, and microbiological quality of food under normal conditions are presented in the following chapters:

Chapter 1: History and Development of Food Microbiology
Chapter 2: Characteristics of Predominant Microorganisms in Food
Chapter 3: Sources of Microorganisms in Food
Chapter 4: Normal Microbiological Quality of Foods and Its Significance

CHAPTER 1

History and Development of Food Microbiology

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I. INTRODUCTION

Except for a few sterile foods, all foods harbor one or more types of microorganisms. Some of them have desirable roles in food, such as in the production of naturally fermented food, whereas others cause food spoilage and foodborne diseases. To study the role of microorganisms in food and to control them when necessary, it is important to isolate them in pure culture and study their morphological, physiological, biochemical, and genetic characteristics. Some of the simplest techniques in use today for these studies were developed over the last 300 years; a brief description is included here.

II. DISCOVERY OF MICROORGANISMS

The discovery of microorganisms[1–3] ran parallel with the invention and improvement of the microscope. Around 1658, Athanasius Kircher reported that, using a microscope, he had seen minute living worms in putrid meat and milk. The magnification power of his microscope was so low that he could not have seen bacteria. In 1664, Robert Hooke described the structure of molds. However, probably the first person to see different types of microorganisms, especially bacteria, under a microscope that probably did not have a magnification power above 300×, was Antony van Leeuwenhoek. He observed bacteria in saliva, rainwater, vinegar, and other materials; sketched the three morphological groups (spheroids or cocci, cylindrical rods or bacilli, and spiral or spirilla); and also described some to be motile. He called them *animalcules*, and between 1676 and 1683 he reported his observations to the newly formed leading scientific organization, The Royal Society of London, where his observations were read with fascination. As reasonably good microscopes were not easily available at the time, other interested individuals and scientists during the next 100 years only confirmed Leeuwenhoek's observations. In the 19th century, as an outcome of the Industrial Revolution, improved microscopes became more easily available, which stimulated many inquisitive minds to observe and describe the creatures they discovered under a microscope. By 1838, Ehrenberg (who introduced the term *bacteria*) had proposed at least 16 species in four genera and by 1875 Ferdinand Cohn had developed the preliminary classification system of bacteria. Cohn also was the first to discover that some bacteria produced spores. Although, like bacteria, the existence of submicroscopic viruses was recognized in the mid-19th century, they were observed only after the invention of the electron microscope in the 1940s.

III. WHERE ARE THEY COMING FROM?

Following Leeuwenhoek's discovery, although there were no bursts of activity, some scientific minds were curious to determine from where the animalcules, observed to be present in many different objects, were emanating.[1–3] Society had just emerged from the Renaissance period, and science, known as experimental philosophy, was in its infancy. The theory of spontaneous generation, i.e., the generation of some form of life from nonliving objects, had many powerful followers among the educated and elite classes. Since the time of the Greeks, the emergence of maggots from dead bodies and spoiled flesh was thought to be due to spontaneous generation. However, ca. 1665, Redi disproved that theory by showing that the maggots in spoiled meat and fish could only appear if flies were allowed to contaminate them. The advocates of the spontaneous generation theory argued that the animalcules could not regenerate by themselves (biogenesis), but they were present in different things only through abiogenesis (spontaneous generation). In 1749, Turbevill Needham showed that boiled meat and meat broth, following storage in covered flasks, could have the presence of animalcules within a short time. This was used to prove the appearance of these animalcules by spontaneous generation. Lazzaro

Spallanzani (1765) showed that boiling the meat infusion in broth in a flask and sealing the flask immediately prevented the appearance of these microscopic organisms, thereby disproving Needham's theory.

This was the time when Antoine Laurent Lavoisier and his coworkers showed the need of oxygen for life. The believers of abiogenesis rejected Spallanzani's observation, suggesting that there was not enough vital force (oxygen) present in the sealed flask for animalcules to appear through spontaneous generation. Later, Schulze (1830, by passing air through acid), Theodore Schwann (1838, by passing air through red-hot tubes), and Schröeder (1854, by passing air through cotton) showed that bacteria failed to appear in boiled meat infusion even in the presence of air. Finally, in 1861, Louis Pasteur demonstrated that, in boiled infusion, bacteria could grow only if the infusions were contaminated with bacteria carried by dust particles in air.[1,4] His careful and controlled studies proved that bacteria were able to reproduce (biogenesis) and life could not originate by spontaneous generation. John Tyndall, in 1870, showed that boiled infusion could be stored in dust-free air in a box without microbial growth.

IV. WHAT ARE THEIR FUNCTIONS?

The involvement of invisible organisms in many diseases in humans was suspected as early as the 13th century by Roger Bacon. In the 16th century, Girolamo Fracastoro of Verona suggested that many human diseases were transmitted from person to person by small creatures. This was also indicated by Kircher in 1658. In 1762, von Plenciz of Vienna suggested that different invisible organisms were responsible for different diseases. Theodore Schwann (1837) and Hermann Helmholtz (1843) proposed that putrefaction and fermentation were connected with the presence of the organisms derived from air. Finally, Pasteur, in 1875, showed that wine fermentation from grapes and souring of wine were caused by microorganisms. He also proved that spoilage of meat and milk was associated with the growth of microorganisms. Later, he showed the association of microorganisms with several diseases in humans, cattle, and sheep, and he also developed vaccines against a few human and animal diseases caused by microorganisms, including rabies. Robert Koch, in Germany (in the 1880s and 1890s), isolated pure cultures of bacteria responsible for anthrax, cholera, and tuberculosis. He also developed the famous Koch's postulates to associate a specific bacterium as a causative agent for a specific disease. Along with his associates, he also developed techniques of agar plating methods to isolate bacteria in pure cultures and to determine microbial numbers in a sample, the Petri dish (by Petri in his laboratory), staining methods for better microscopic observation of bacteria, and the use of steam to sterilize materials to grow bacteria.[1,5]

With time, the importance of microorganisms in human and animal diseases, soil fertility, plant diseases, fermentation, food spoilage and foodborne diseases, and other areas was recognized, and microbiology was developed as a specific discipline. Later, it was divided into several subdisciplines, such as medical microbiology, soil microbiology, plant pathology, and food microbiology.

V. DEVELOPMENT OF EARLY FOOD MICROBIOLOGY (BEFORE 1900 A.D.)

It is logical to comprehend that our early *Homo* ancestors, the hunters and gatherers, were aware of food spoilage and foodborne diseases. Even without any perception of the causative agents, they used ice and fire to preserve foods and make them safe. Around 8000 B.C., as agriculture and animal husbandry were adopted by the early civilizations, food supply, especially agricultural produce, became available in abundance during the growing seasons. Preservation of foods became important for uniform supply of food around the year. Between 8000 and 1000 B.C., many food preservation methods such as drying, cooking, baking, smoking, salting, sugaring (with honey), low-temperature storage (in ice), storage without air (in pits), fermentation (with fruits, grains, and milk), pickling, and spicing were used, probably mainly to reduce spoilage. However, one cannot be sure whether the society at that time recognized the implications of diseases transmitted through food. In the later periods, however, the scriptural injunctions laid by many religions suggest that the societies recognized an association of diseases with some foods. Some of the regulations, such as not eating meat from a diseased animal or an animal killed by a scavenger, or not eating a food that appeared unnatural or had been handled by an unclean person, were developed to safeguard the health of citizens against foodborne diseases. Fermentation was used extensively by many societies not only to preserve foods but also as a method to produce various types of desirable foods from milk, meat, fish, eggs, grains, fruits, and vegetables.

Following the discovery of the ubiquitous existence of microorganisms (mainly bacteria and yeasts) by Leeuwenhoek around the 1670s, some individuals started associating the possible role of these organisms with food spoilage, food fermentation, and foodborne diseases. The major developments of ideas on the possible roles of microorganisms in foods and their scientific proof were initiated by Pasteur in the 1870s, followed by many other scientists before the end of the 19th century. This paved the way for the establishment of early food microbiology in the 20th century. Some of the major developments in the 19th century are briefly listed here.[1,6,7]

	Food Fermentation
1822	C.J. Person named the microscopic organism found on the surface of wine during vinegar production as *Mycoderma mesentericum*. Pasteur in 1868 proved that this organism was associated with the conversion of alcohol to acetic acid and named it *Mycoderma aceti*. In 1898, Martinus Beijerinck renamed it *Acetobacter aceti*.
1837	Theodor Schwann named the organism involved in sugar fermentation as *Saccharomyces* (sugar fungus).
1838	Charles Cogniard-Latour suggested that growth of yeasts was associated with alcohol fermentation.
1860	Louis Pasteur showed that fermentation of lactic acid and alcohol from sugar was the result of growth of specific bacteria and yeasts, respectively.
1883	Emil Christian Hansen used pure cultures of yeasts to ferment beer.

	Food Spoilage
1804	Francois Nicolas Appert developed methods to preserve foods in sealed glass bottles by heat in boiling water. He credited this process to Lazzaro Spallanzani (1765), who first used the method to disprove the spontaneous generation theory.
1819	Peter Durand developed canning preservation of foods in steel cans. Charles Mitchell introduced tin lining of metal cans in 1839.
1870	L. Pasteur recommended heating of wine at 145°F (62.7°C) for 30 min to destroy souring bacteria. F. Soxhlet advanced boiling of milk for 35 min to kill contaminated bacteria. Later, this method was modified and named pasteurization, and used to kill mainly vegetative pathogens and many spoilage bacteria.
1895	Harry Russell showed that gaseous swelling with bad odors in canned peas was due to growth of heat-resistant bacteria (spores).

	Foodborne Diseases
1820	Justin Kerner described food poisoning from eating blood sausage (due to botulism). Fatal disease from eating blood sausage was recognized as early as A.D. 900.
1849	John Snow suggested the spread of cholera through drinking water contaminated with sewage. In 1854, Filippo Facini named the cholera bacilli as *Vibrio cholera*, which was isolated in pure form by Robert Koch in 1884.
1856	William Budd suggested that water contamination with feces from infected person spread typhoid fever and advocated the use of chlorine in water supply to overcome the problem. In 1800, G. de Morveau and W. Cruikshank advocated the use of chlorine to sanitize potable water.
1885	Theodor Escherich isolated *Bacterium coli* (later named *Escherichia coli*) from the feces and suggested that some strains were associated with infant diarrhea.
1888	A.A. Gartner isolated *Bacterium* (later *Salmonella*) *enteritidis* from the organs of a diseased man as well as from the meat the man ate. In 1896, Marie von Ermengem proved that *Salmonella enteritidis* caused a fatal disease in humans who consumed contaminated sausage.
1894	J. Denys associated pyogenic *Staphylococcus* with death of a person who ate meat prepared from a diseased cow.
1895	Marie von Ermengem isolated *Bacillus botulinus* (*Clostridium botulinum*) from contaminated meat and proved that it caused botulism.

	Microbiology Techniques
1854	Heinrich Schröder and Theodore von Dusch used cotton to close tubes and flasks to prevent microbial contamination in heated culture broths.
1876	Car Weigert used methylene blue (a synthetic dye) to stain bacteria in aqueous suspensions.
1877	Ferdinand Cohn showed heat resistance of *Bacillus subtilis* endospores.
1878	Joseph Lister isolated *Streptococcus* (now *Lactococcus*) *lactis* in pure culture by serial dilution from sour milk.
1880s	Robert Koch and his associates introduced many important methods that are used in all branches of microbiology, such as solid media (first gelatin, then agar) to purify and enumerate bacteria, Petri dish, flagellar staining, steam sterilization of media above 100°C, and photography of cells and spores.
1884	Hans Christian Gram developed Gram staining of bacterial cells.

VI. FOOD MICROBIOLOGY: CURRENT STATUS

In the early 20th century, studies continued to understand the association and importance of microorganisms, especially pathogenic bacteria in food. Specific methods were developed for their isolation and identification. The importance of sanitation in the handling of food to reduce contamination by microorganisms was recognized. Specific methods were studied to prevent growth as well as to destroy the spoilage and pathogenic bacteria. There was also some interest to isolate beneficial bacteria associated with food fermentation, especially dairy fermentation, and study their characteristics. However, after the 1950s, food microbiology entered a new era. Availability of basic information on the physiological, biochemical, and biological characteristics of diverse types of food, microbial interactions in food environments and microbial physiology, biochemistry, genetics, and immunology has helped open new frontiers in food microbiology. Among these are: [1,6–8]

A. Food Fermentation/Probiotics

- Development of strains with desirable metabolic activities by genetic transfer among strains
- Development of bacteriophage-resistant lactic acid bacteria
- Metabolic engineering of strains for overproduction of desirable metabolites
- Development of methods to use lactic acid bacteria to deliver immunity proteins
- Sequencing genomes of important lactic acid bacteria and bacteriophages for better understanding of their characteristics
- Food biopreservation with desirable bacteria and their antimicrobial metabolites
- Understanding of important characteristics of probiotic bacteria and development of desirable strains
- Effective methods to produce starter cultures for direct use in food processing

B. Food Spoilage

- Identification and control of new spoilage bacteria associated with the current changes in food processing and preservation methods
- Spoilage due to bacterial enzymes of frozen and refrigerated foods with extended shelf life
- Development of molecular methods (nanotechnology) to identify metabolites of spoilage bacteria and predict potential shelf life of foods
- Importance of environmental stress on the resistance of spoilage bacteria to antimicrobial preservatives

C. Foodborne Diseases

- Methods to detect emerging foodborne pathogenic bacteria from contaminated foods
- Application of molecular biology techniques (nanotechnology) for rapid detection of pathogenic bacteria in food and environment
- Effective detection and control methods of foodborne pathogenic viruses
- Transmission potentials of prion diseases from food animals to humans

- Importance of environmental stress on the detection and destruction of pathogens
- Factors associated with the increase in antibiotic-resistant pathogens in food
- Adherence of foodborne pathogens on food and equipment surfaces
- Mechanisms of pathogenicity of foodborne pathogens
- Effective methods for epidemiology study of foodborne diseases
- Control of pathogenic parasites in food

D. Miscellaneous

- Application of hazard analysis of critical control points (HACCP) in food production, processing, and preservation
- Novel food-processing technologies
- Microbiology of unprocessed and low-heat-processed ready-to-eat foods
- Microbial control of foods from farm to table (total quality management)
- Food safety legislation

VII. FOOD MICROBIOLOGY AND FOOD MICROBIOLOGISTS

From the above discussion, it is apparent what, as a discipline, food microbiology has to offer. Before the 1970s, food microbiology was regarded as an applied science mainly involved in the microbiological quality control of food. Since then, the technology used in food production, processing, distribution and retailing and food consumption patterns have changed dramatically. These changes have introduced new problems that can no longer be solved by merely using applied knowledge. Thus, modern-day food microbiology needs to include a great deal of basic science to understand and effectively solve the microbiological problems associated with food. The discipline includes not only microbiological aspects of food spoilage and foodborne diseases and their effective control and bioprocessing of foods but also basic information of microbial ecology, physiology, metabolism, and genetics. This information is helping to develop methods for rapid and effective detection of spoilage and pathogenic bacteria, to develop desirable microbial strains by recombinant DNA technology, to produce fermented foods of better quality, to develop thermostable enzymes in enzyme processing of food and food additives, to develop methods to remove bacteria from food and equipment surfaces, and to combine several control methods for effective control of spoilage and pathogenic microorganisms in food.

An individual who has completed courses in food microbiology (both lecture and laboratory) should gain knowledge in the following areas:

- Determine microbiological quality of foods and food ingredients by using appropriate techniques
- Determine microbial types involved in spoilage and health hazards and identify the sources
- Design corrective procedures to control the spoilage and pathogenic microorganisms in food
- Learn rapid methods to isolate and identify pathogens and spoilage bacteria from food and environment

- Identify how new technologies adapted in food processing can have specific microbiological problems and design methods to overcome the problem
- Design effective sanitation procedures to control spoilage and pathogen problems in food-processing facilities
- Effectively use desirable microorganisms to produce fermented foods
- Design methods to produce better starter cultures for use in fermented foods and probiotics
- Know about food regulations (state, federal, and international)
- Understand microbiological problems of imported foods

To be effective, in addition to the knowledge gained, one has to be able to communicate with different groups of people about the subject (food microbiology and its relation to food science). An individual with good common sense is always in a better position to sense a problem and correct it quickly.

VIII. CONCLUSION

The human civilization began when hunters and gatherers adopted not only production but also preservation of foods. Thus, long before the existence of microorganisms was discovered, their importance on food spoilage and health hazard were conceived by our early ancestors. Once their association and importance in food were proven, efforts were made to understand the basic principles associated with food and microbial interactions. This knowledge was used to control undesirable microbes and effectively use the desirable types. Current investigations are directed toward understanding microbes at the molecular level. A food microbiologist should have a good understanding of the current developments in food microbiology as well as the characteristics of microorganisms important in food. The latter aspect is discussed in Chapter 2.

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QUESTIONS

1. Describe briefly the contributions of the following scientists in the development of microbiology and food microbiology: (a) Leeuwenhoek, (b) Spallanzani, (c) Pasteur, (d) Koch, (e) Hess, (f) Cohn, (g) Lister, (h) Soxhlet (i) Gartner, and (j) Appert.
2. Why did Needham's experiments fail to disprove spontaneous generation for microbes, but Pasteur succeed in disproving that theory?
3. List three important areas (each) of current studies in food biotechnology, food spoilage, and foodborne diseases.
4. Briefly explain the major differences in the understanding of the importance of microorganisms in foods before and after the 1900s.
5. List the pathogens that were proven to be associated with foodborne diseases before 1900.
6. Briefly describe what a food microbiologist student is expected to know.

CHAPTER 2

Characteristics of Predominant Microorganisms in Food

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I. INTRODUCTION

The microbial groups important in foods consist of several species and types of bacteria, yeasts, molds, and viruses. Although some algae and protozoa as well as some worms (such as nematodes) are important in foods, they are not included among the microbial groups in this chapter. Some of the protozoa and worms associated with health hazards, and several algae associated with health hazards and bioprocessing (sources of vitamins, single-cell proteins), are discussed in Chapter 16 and Chapter 27.

Bacteria, yeasts, molds, and viruses are important in food for their ability to cause foodborne diseases and food spoilage and to produce food and food ingredients. Many bacterial species and some molds and viruses, but not yeasts, are able to cause foodborne diseases. Most bacteria, molds, and yeasts, because of their ability to grow in foods (viruses cannot grow in foods), can potentially cause food spoilage. Several species of bacteria, molds, and yeasts are considered safe or food grade, or both, and are used to produce fermented foods and food ingredients. Among the four major groups, bacteria constitute the largest group. Because of their ubiquitous presence and rapid growth rate, even under conditions where yeasts and molds cannot grow, they are considered the most important in food spoilage and foodborne diseases.

Prion or proteinaceous infectious particles have recently been identified to cause transmissible spongiform encephalopathies (TSEs) in humans and animals. However, their ability to cause foodborne diseases is not clearly understood.

In this chapter, a brief discussion is included initially on the methods currently used in the classification and nomenclature of microorganisms and later on the important characteristics of microorganisms predominant in food.

II. CLASSIFICATION OF MICROORGANISMS

Living cellular organisms, on the basis of phylogenetic and evolutionary relationships, were grouped originally in five kingdoms, in which bacteria belonged to procaryotes (before nucleus) and the eucaryotic (with nucleus) molds and yeasts were grouped under fungi.[1–3] In the 1970s, the procaryotic domain was changed to Eubacteria (with murine on cell wall) and Archaebacteria (without murine on cell wall). In the 1990s, this was changed to Bacteria and Archaea, respectively.[4] Archaea include most extremophiles and are not important to food microbiology. Viruses are not considered as living cells and are not included in this classification system.

For the classification of yeasts, molds, and bacteria, several ranks are used after the kingdom: divisions, classes, orders, families, genera (singular genus), and species. The basic taxonomic group is the species. Several species with similar characteristics form a genus. Among eucaryotes, species in the same genus can interbreed. This is not considered among procaryotes, although conjugal transfer of genetic materials exists among many bacteria. Several genera make a family, and the same procedure is followed in the hierarchy. In food microbiology, ranks above species, genus, and family are seldom used. Among bacteria, a species is regarded as a collection of strains having many common features. A strain is the descendent of a single colony (single cell). Among the strains in a species, one is assigned as the type strain, and is used as a reference strain while comparing the characteristics of an unknown isolate. However, by knowing the complete genome sequence, this system will change in the future.

Several methods are used to determine relatedness among bacteria, yeasts, and molds for taxonomic classification. In yeasts and molds, morphology, reproduction, biochemical nature of macromolecules, and metabolic patterns are used along with other criteria. For bacterial morphology, Gram-stain characteristics, protein profiles, amino acid sequences of some specific proteins, base composition (mol% G + C), nucleic acid (DNA and RNA) hybridization, nucleotide base sequence, and computer-assisted numerical taxonomy are used.[1–3] Protein profile, amino acid sequence, base composition, DNA and RNA hybridization, and nucleotide base sequence are directly or indirectly related to genetic makeup of the organisms and thus provide a better chance in comparing two organisms at the genetic level. In mol% G + C ratio, if two strains differ by 10% or more, they are most likely not related. Similarly, in a hybridization study, two strains are considered the same if their DNAs have 90% or more homology. For the nucleotide base sequence, the sequences in 16S rRNA among strains are compared. A sequence of about 1500 nucleotide bases over a stretch of 16S rRNA is most conserved, so related strains should have high homology. In numerical taxonomy, many characteristics are compared, such as morphological, physiological, and biochemical. Each characteristic is given the same weightage. Two strains in the same species should score 90% or more.

Evolutionary relationships among viruses, if any, are not known. Their classification system is rather arbitrary and based on the types of disease they cause (such as the hepatitis virus, causing inflammation of the liver), nucleic acid content (RNA or DNA, single stranded or double stranded), and morphological structures. In food, two groups of viruses are important: the bacterial viruses (bacteriophages) of starter culture bacteria and some foodborne pathogenic bacteria, and the human pathogenic viruses associated with foodborne diseases.

III. NOMENCLATURE

The basic taxonomic group in bacteria, yeasts, and molds is the species, and each species is given a name.[1–3] The name has two parts (binomial name): the first part is the genus name and the second part is the specific epithet (adjective). Both parts are Latinized; when written, they are italicized (or underlined), with the first letter of the genus written in a capital letter (e.g., *Saccharomyces cerevisiae, Penicillium roquefortii,* and *Lactobacillus acidophilus*). A bacterial species can be divided into several subspecies (subsp. or ssp.) if the members show minor but consistent differences in characteristics. Under such conditions, a trinomial epithet (subspecific epithet) is used (e.g., *Lactococcus lactis* ssp. *lactis* or *Lactococcus lactis* ssp. *cremoris*). In some instances, ranks below subspecies are used to differentiate strains recognized by specific characters (e.g., serovar, antigenic reaction; biovar, producing a specific metabolite; and phagovar, sensitive to a specific phage). Such ranks have no taxonomic importance but can be practically useful (e.g., *Lactococcus lactis* ssp. *lactis* biovar diacetilactis is a *Lactococcus lactis* ssp. *lactis* strain that produces diacetyl, an important flavor compound in some fermented dairy products). Each strain of a species should be identified with a specific strain number, which can be alphabetic or numeric or a mixture of both (e.g., *Pediococcus acidilactici* LB923). At the family level, bacterial names are used as plural adjectives in feminine gender and agree with the suffix "aceae" (e.g., *Enterobacteriaceae*). The species and strains in a genus can be represented collectively, either using "spp." after genus (e.g., *Lactobacillus* spp.) or plural forms of the genus (e.g., lactobacilli for *Lactobacillus*; lactococci for *Lactococcus*; leuconostocs for *Leuconostoc*, or salmonellae for *Salmonella*).

The scientific names of bacteria are given according to the specifications of the International Code of Nomenclature of Bacteria. The International Committee on Systematic Bacteriology of the International Union of Microbiological Association examines the validity of each name and then publishes the approved lists of bacterial names from time to time. A new name (species or genus) must be published in the *International Journal of Systematic Bacteriology* before it is judged for inclusion in the approved list. Any change in name (genus or species) has to be approved by this committee.

When writing the name of the same species more than once in an article, it is customary to use both genus and specific epithet the first time and abbreviate the genus name subsequently. In the *Bergey's Manual of Systematic Bacteriology*, only the first letter is used (e.g., *Listeria monocytogenes* and then *L. monocytogenes*). The same system is used in most publications in the U.S. However, it creates

confusion when one article has several species with the same first letter in the genus (e.g., *Lactobacillus lactis, Leuconostoc lactis,* and *Lactococcus lactis* as *L. lactis*). In some European journals, more than one letter is used, but there is no definite system (e.g., *Lact. lactis, Lc. lactis, Leu. lactis, Lb. lactis, List. monocytogenes*). In this book, to reduce confusion among readers, many of whom might not be familiar with the current rapid changes in bacterial nomenclature, a three-letter system is used (e.g., *Lis. monocytogenes, Leu. lactis; Sal. typhimurium* for *Salmonella; Shi. dysenterie* for *Shigella; Sta. aureus* for *Staphylococcus*). In rare cases, a slight modification is used (e.g., *Lactococcus lactis* and *Lactobacillus lactis* are written as *Lac. lactis* and *Lab. lactis*, respectively, for the two genera). Recently, the nomenclature system for *Salmonella* has been modified (Chapter 25).

The viruses, as indicated previously, have not been given specific taxonomic names as given for bacteria. They are often identified with alphabetic or numeric designation, or a combination of both (e.g., T4 or λ bacteriophages), the disease they produce (e.g., hepatitis A, causing liver inflammation), or by other methods (e.g., Norwalk-like viruses, causing a type of foodborne gastroenteritis in humans).

IV. MORPHOLOGY AND STRUCTURE OF MICROORGANISMS IN FOODS

A. Yeasts and Molds

Both yeasts and molds are eucaryotic, but yeasts are unicellular whereas molds are multicellular.[5] Eucaryotic cells are generally much larger (20 to 100 μm) than procaryotic cells (1 to 10 μm). Eucaryotic cells have rigid cell walls and thin plasma membranes. The cell wall does not have mucopeptide, is rigid, and is composed of carbohydrates. The plasma membrane contains sterol. The cytoplasm is mobile (streaming) and contains organelles (mitochondria, vacuoles) that are membrane bound. Ribosomes are 80S type and attached to the endoplasmic reticulum. The DNA is linear (chromosomes), contains histones, and is enclosed in a nuclear membrane. Cell division is by mitosis (i.e., asexual reproduction); sexual reproduction, when it occurs, is by meiosis.

Molds are nonmotile, filamentous, and branched (Figure 2.1). The cell wall is composed of cellulose, chitin, or both. A mold (thallus) is composed of large numbers of filaments called hyphae. An aggregate of hyphae is called mycelium. A hypha can be nonseptate, septate-uninucleate, or septate-multinucleate. A hypha can be vegetative or reproductive. The reproductive hypha usually extends in the air and form exospores, either free (conidia) or in a sack (sporangium). Shape, size, and color of spores are used for taxonomic classification.

Yeasts are widely distributed in nature. The cells are oval, spherical, or elongated, about 5–30 × 2–10 μm in size (Figure 2.1).[5,6] They are nonmotile. The cell wall contains polysaccharides (glycans), proteins, and lipids. The wall can have scars, indicating the sites of budding. The membrane is beneath the wall. The cytoplasm has a finely granular appearance for ribosomes and organelles. The nucleus is well-defined with a nuclear membrane.

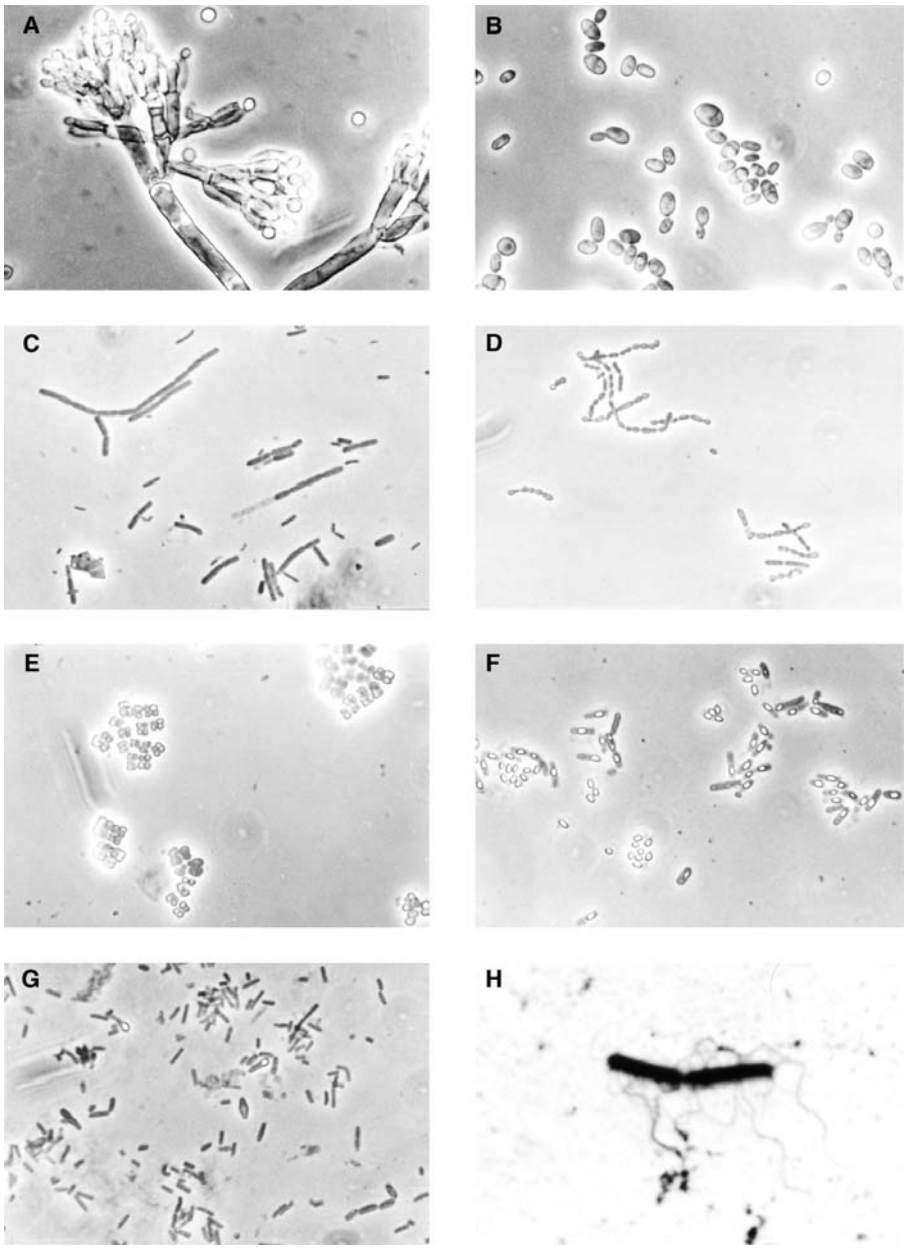


Figure 2.1 Photograph of microbial morphology. (A) Molds: Conidial head of *Penicillium* sp. showing conidiophore (stalk) and conidia. (B) Yeasts: *Saccharomyces cerevisiae*, some carrying buds. (C) Rod-shaped bacteria: *Bacillus* sp., single and chain. (D) Spherical-shaped bacteria: *Streptococcus* sp., chain. (E) Spherical-shaped bacteria: tetrads. (F) *Bacillus* cells carrying spores, center and off-center. (G) *Clostridium* cells, some carrying terminal spore (drumstick appearance). (H) Motile rod-shaped bacterium (*Clostridium* sp.) showing peretrichous flagella.

B. Bacterial Cells

Bacteria are unicellular, most ca. 0.5–1.0 × 2.0–10 μm in size, and have three morphological forms: spherical (cocci), rod shaped (bacilli), and curved (comma) (Figure 2.1).[7] They can form associations such as clusters, chains (two or more cells), or tetrads. They can be motile or nonmotile. Cytoplasmic materials are enclosed in a rigid wall on the surface and a membrane beneath the wall. Nutrients in molecular and ionic form are transported from the environment through the membrane by several but specific mechanisms. The membrane also contains energy-generating components. It also forms intrusions in the cytoplasm (mesosomes). The cytoplasmic material is immobile and does not contain organelles enclosed in a separate membrane. The ribosomes are 70S type and are dispersed in the cytoplasm. The genetic materials (structural and plasmid DNA) are circular, not enclosed in nuclear membrane, and do not contain basic proteins such as histones. Both gene transfer and genetic recombination occur, but do not involve gamete or zygote formation. Cell division is by binary fission. Procaryotic cells can also have flagella, capsules, surface layer proteins, and pili for specific functions. Some also form endospores (one per cell).

On the basis of Gram-stain behavior, bacterial cells are grouped as Gram-negative or Gram-positive. Gram-negative cells have a complex cell wall containing an outer membrane (OM) and a middle membrane (MM) (Figure 2.2). The OM is composed of lipopolysaccharides (LPS), lipoprotein (LP), and phospholipids. Phospholipid molecules are arranged in a bilayer, with the hydrophobic part (fatty acids) inside and hydrophilic part (glycerol and phosphate) outside. LPS and LP molecules are embedded in the phospholipid layer. The OM has limited transport and barrier functions. The resistance of Gram-negative bacteria to many enzymes (lysozyme, which hydrolyzes mucopeptide), hydrophobic molecules (SDS and bile salts), and antibiotics (penicillin) is due to the barrier property of the OM. LPS molecules also have antigenic properties. Beneath the OM is the MM, composed of a thin layer of peptidoglycan or mucopeptide embedded in the periplasmic materials that contain several types of proteins. Beneath the periplasmic materials is the plasma or inner membrane (IM), composed of a phospholipid bilayer in which many types of proteins are embedded.

Gram-positive cells have a thick cell wall composed of several layers of mucopeptide (responsible for thick rigid structure) and two types of teichoic acids (Figure 2.2). Some species also have a layer over the cell surface, called surface layer protein (SLP). The wall teichoic acid molecules are linked to mucopeptide layers, and the lipoteichoic acid molecules are linked to both mucopeptide and cytoplasmic membrane. Teichoic acids are negatively charged (because of phosphate groups) and may bind to or regulate the movement of cationic molecules in and out of the cell. Teichoic acids have antigenic properties and can be used to identify Gram-positive bacteria serologically. Because of the complexity in the chemical composition of the cell wall, Gram-positive bacteria are considered to have evolved before Gram-negative bacteria.

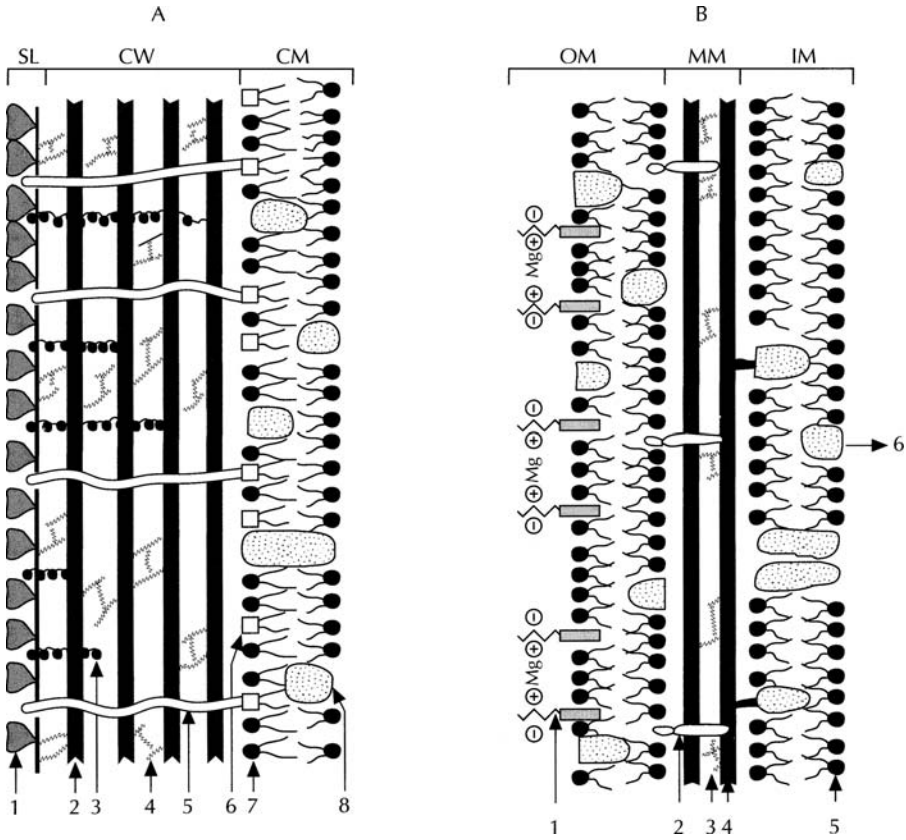


Figure 2.2 Schematic representations of cell envelopes of bacteria. (A) Gram-positive bacteria: SL: surface layer proteins with protein subunits (1); CW: cell wall showing thick mucopeptide backbone layers (2) covalently linked to peptides (4), wall teichoic acids (or teichouronic acid); (3), lipoteichoic acids (anchored to cytoplasmic membrane; (5); CM: cytoplasmic membrane with lipid bilayers containing phospholipids (7), glycolipids (6) and embedded proteins (8). (B) Gram-negative bacteria: OM: outer membrane containing lipopolysaccharide molecules, stabilized by divalent cations (1), phospholipids and proteins; MM, middle membrane containing thin mucopeptide layers (4) covalently liked to peptides (3) and lipoproteins (2); IM: inner membrane with phospholipid bilayers (5) and proteins (6).

C. Viruses

Viruses are regarded as noncellular entities. Bacterial viruses (bacteriophages) important in food microbiology are widely distributed in nature.[8–10] They are composed of nucleic acids (DNA or RNA) and several proteins. The proteins form the head (surrounding the nucleic acid) and tail.

A bacteriophage attaches itself to the surface of a host bacterial cell and inoculates its nucleic acid into the host cell. Subsequently, many phages form inside a host cell and are released outside following lysis of the cell. This is discussed in Chapter 13.

Several pathogenic viruses have been identified as causing foodborne diseases in humans. However, because they are difficult to detect in foods, the involvement of other pathogenic viruses in foodborne diseases is not correctly known. The two most important viruses implicated in foodborne outbreaks are hepatitis A and Norwalk-like viruses. Both are single-stranded RNA viruses. Hepatitis A is a small, naked, polyhedral enteric virus ca. 30 nm in diameter. The RNA strand is enclosed in a capsid.

V. IMPORTANT MICROORGANISMS IN FOOD

A. Important Mold Genera

Molds are important in food because they can grow even in conditions in which many bacteria cannot grow, such as low pH, low water activity (A_w), and high osmotic pressure. Many types of molds are found in foods.[5] They are important spoilage microorganisms. Many strains also produce mycotoxins and have been implicated in foodborne intoxication. Many are used in food bioprocessing. Finally, many are used to produce food additives and enzymes. Some of the most common genera of molds found in food are listed here (also see Figure 8.1).

Aspergillus. It is widely distributed and contains many species important in food. Members have septate hyphae and produce black-colored asexual spores on conidia. Many are xerophilic (able to grow in low A_w) and can grow in grains, causing spoilage. They are also involved in spoilage of foods such as jams, cured ham, nuts, and fruits and vegetables (rot). Some species or strains produce mycotoxins (e.g., *Aspergillus flavus* produces aflatoxin). Many species or strains are also used in food and food additive processing. *Asp. oryzae* is used to hydrolyze starch by α-amylase in the production of sake. *Asp. niger* is used to process citric acid from sucrose and to produce enzymes such as β-galactosidase.

Alternaria. Members are septate and form dark-colored spores on conidia. They cause rot in tomatoes and rancid flavor in dairy products. Some species or strains produce mycotoxins. Species: *Alternaria tenuis*.

Fusarium. Many types are associated with rot in citrus fruits, potatoes, and grains. They form cottony growth and produce septate, sickle-shaped conidia. Species: *Fusarium solani*.

Geotrichum. Members are septate and form rectangular arthrospores. They grow, forming a yeastlike cottony, creamy colony. They establish easily in equipment and often grow on dairy products (dairy mold). Species: *Geotrichum candidum*.

Mucor. It is widely distributed. Members have nonseptate hyphae and produce sporangiophores. They produce cottony colonies. Some species are used in food fermentation and as a source of enzymes. They cause spoilage of vegetables. Species: *Mucor rouxii*.

Penicillium. It is widely distributed and contains many species. Members have septate hyphae and form conidiophores on a blue-green, brushlike conidia head (Figure 2.1). Some species are used in food production, such as *Penicillium roquefortii* and *Pen. camembertii* in cheese. Many species cause fungal rot in fruits and

vegetables. They also cause spoilage of grains, breads, and meat. Some strains produce mycotoxins (e.g., Ochratoxin A).

Rhizopus. Hyphae are aseptate and form sporangiophores in sporangium. They cause spoilage of many fruits and vegetables. *Rhizopus stolonifer* is the common black bread mold.

B. Important Yeast Genera

Yeasts are important in food because of their ability to cause spoilage. Many are also used in food bioprocessing. Some are used to produce food additives. Several important genera are briefly described next.[6]

Saccharomyces. Cells are round, oval, or elongated. It is the most important genus and contains heterogenous groups (Figure 2.1). *Saccharomyces cerevisiae* variants are used in baking for leavening bread and in alcoholic fermentation. They also cause spoilage of food, producing alcohol and CO_2.

Pichia. Cells are oval to cylindrical and form pellicles in beer, wine, and brine to cause spoilage. Some are also used in oriental food fermentation. Species: *Pichia membranaefaciens*.

Rhodotorula. They are pigment-forming yeasts and can cause discoloration of foods such as meat, fish, and sauerkraut. Species: *Rhodotorula glutinis*.

Torulopsis. Cells are spherical to oval. They cause spoilage of milk because they can ferment lactose (e.g., *Torulopsis versatilis*). They also spoil fruit juice concentrates and acid foods.

Candida. Many species spoil foods with high acid, salt, and sugar and form pellicles on the surface of liquids. Some can cause rancidity in butter and dairy products (e.g., *Candida lipolyticum*).

Zygosaccharomyces. Cause spoilage of high-acid foods, such as sauces, ketchups, pickles, mustards, mayonnaise, salad dressings, especially those with less acid and less salt and sugar (e.g., *Zygosaccharomyces bailii*).

C. Important Viruses

Viruses are important in food for three reasons.[8–10] Some are able to cause enteric disease, and thus, if present in a food, can cause foodborne diseases. Hepatitis A and Norwalk-like viruses have been implicated in foodborne outbreaks. Several other enteric viruses, such as poliovirus, echo virus, and Coxsackie virus, can cause foodborne diseases. In some countries where the level of sanitation is not very high, they can contaminate foods and cause disease.

Some bacterial viruses (bacteriophages) are used to identify some pathogens (*Salmonella* spp., *Staphylococcus aureus* strains) on the basis of the sensitivity of the cells to a series of bacteriophages at appropriate dilutions. Bacteriophages are used to transfer genetic traits in some bacterial species or strains by a process called transduction (e.g., in *Escherichia coli* or *Lactococcus lactis*).

Finally, some bacteriophages can be very important because they can cause fermentation failure. Many lactic acid bacteria, used as starter cultures in food fermentation, are sensitive to different bacteriophages. They can infect and destroy starter-culture bacteria, causing product failure. Among the lactic acid bacteria,

bacteriophages have been isolated for many species in the genera *Lactococcus, Streptococcus, Leuconostoc,* and *Lactobacillus*; no bacteriophage of *Pediococcus* is yet known. Methods are being devised to genetically engineer lactic starter cultures so that they become resistant to multiple bacteriophages (see Chapter 13).

D. Important Bacterial Genera

Bacterial classification is changing rapidly.[1–3] In *Bergey's Manual of Systematic Bacteriology*, published between 1984 and 1988, more than 420 bacterial genera are listed in 33 sections on the basis of their differences in characteristics. Since then, many other genera have been created, such as *Lactococcus* (former N-group or dairy *Streptococcus*) and *Carnobacterium* (some species previously included in *Lactobacillus*). In the ninth edition of *Bergey's Manual of Determinative Bacteriology* (1993), more than 560 genera are listed in 35 groups. Of these, Table 2.1 lists 48 genera whose species are frequently associated with spoilage, health hazard, and bioprocessing of food. Species of other genera besides these 48 can also be found in food, but their relative significance is not well established. Many species names in several genera are also no longer valid and thus not included in the current *Bergey's Manual of Determinative Bacteriology.* In this text, only species and genera currently approved and listed in *Bergey's Manual* are used. Brief important characteristics of these genera and their importance in foods are described. Some descriptions are also presented in other chapters, such as pathogens in Chapter 24 to Chapter 26 and beneficial bacteria (bioprocessing) in Chapter 10 and Chapter 17.

Since the publication of the ninth edition of *Bergey's Manual of Determinative Bacteriology*, many other new genera have been created. A few that are important in food are listed separately in this chapter. The second edition of *Bergey's Manual of Systematic Bacteriology* is being published in five volumes. Once they are published, within the next two to four years, better information will be available on bacterial genera and species important in food.

1. Gram-Negative Aerobes

Campylobacter. Two species, *Campylobacter jejuni* and *Cam. coli*, are foodborne pathogens. Small (0.2×1 μm) microaerophilic, helical, motile cells found in the intestinal tract of humans, animals, and birds. Mesophiles.

Pseudomonas. Straight or curved (0.5×5 μm); aerobes; motile rods; psychrotrophs (grow at low temperatures). Found widely in the environment. Includes large numbers of species. Some important species in foods are *Pseudomonas fluorescens, Pse. aeruginosa,* and *Pse. putida.* Important spoilage bacteria, can metabolize a wide variety of carbohydrates, proteins, and lipids in foods.

Xanthomonas. Most characteristics of this group are similar to those for *Pseudomonas*. Plant pathogens, can thus cause spoilage of fruits and vegetables. *Xanthomonas campestris* strains used to produce xanthan gum, which is used as a food stabilizer.

Acetobacter. Ellipsoid to rod-shaped (0.6×4 μm); occur singly or in short chains; motile or nonmotile; aerobes; oxidize ethanol to acetic acid; mesophiles. Cause souring of alcoholic beverages and fruit juices and used to produce vinegar (acetic

Table 2.1 Genera of Bacteria Important in Foods

Section[a](Group[b])	Description	Family	Genera
2	Gram-negative, aerobic/microaerophilic, motile, helical/vibrioid	Not indicated	*Campylobacter, Arcobacter, Helicobacter*[c]
4	Gram-negative, aerobic, rods and cocci	*Pseudomonadaceae*	*Pseudomonas,Xanthomonas*
		Acetobacteraceae	*Acetobacter, Gluconobacter*
		Nisseriaceae	*Acinetobacter, Morexella*
		Not indicated	*Alteromonas, Flavobacterium, Alcaligenes, Brucella, Psychrobacter*
5	Gram-negative, facultative anaerobic, rods	*Enterobacteriaceae*	*Citrobacter, Escherichia, Enterobacter, Edwardsiella, Erwinia, Hafnia, Klebsiella, Morganella, Proteus,Salmonella, Shigella, Serratia, Yersinia*
		Vibrionaceae	*Vibrio, Aeromonas, Plesiomonas*
9	Rickettsias	*Rickettsiaceae*	*Coxiella*
12 (17)	Gram-positive, cocci	*Micrococcaceae*	*Micrococcus, Staphylococcus*
		Not indicated	*Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Sarcina*
13 (18)	Gram-positive, endospore-forming rods and cocci	Not indicated	*Bacillus, Sporolactobacillus, Clostridium,* (*Desulfotomaculum*[d])
14 (19)	Gram-positive, nonsporing, regular rods	Not indicated	*Lactobacillus, Carnobacterium, Brochothrix, Listeria*
15 (20)	Gram-positive, nonsporing, irregular rods	Not indicated	*Corynebacterium, Brevibacterium, Propionibacterium, Bifidobacterium*

[a] Sections in *Bergey's Manual of Systematic Bacteriology.*
[b] Groups in *Bergey's Manual of Determinative Bacteriology.* Only those sections (or groups) containing bacteria important in food are listed in this table.
[c] Are included in this group and contain pathogenic species that can be foodborne.
[d] *Disulfotomaculum* cells stain Gram-negative.

acid). Can also spoil some fruits (rot). Widely distributed in plants and in places where alcohol fermentation occurs. Important species: *Acetobacter aceti*.

Gluconobacter. Many characteristics of this group similar to those of *Acetobacter. Gluconobacter oxydans* causes spoilage of pineapples, apples, and pears (rot).

Acinetobacter. Rods (1×2 μm); occur in pairs or small chains; show twitching motility because of the presence of polar fimbriae; strictly aerobic and grow between 20 and 35°C. Found in soil, water, and sewage. Important species: *Acinetobacter calcoaceticus*.

Morexella. Very short rods, frequently approaching coccoid shape (1×1.5 μm); occur singly, in pairs, or short chains; may be capsulated; twitching motility may be present in some cells; optimum growth at 30 to 35°C. Found in the mucous membrane of animals and humans. Important species: *Morexella lacunata*.

Alteromonas. Most currently assigned *Alteromonas* species are of marine origin and might be present in foods of marine origin. Need 100 mM NaCl for optimum growth (unlike *Pseudomonas*). Because *Alteromonas putrefacience* (species recently reclassified as *Shewanella putrifacience*) has many characters similar to those of *Pseudomonas*, it was previously designated as *Pseudomonas putrefacience*. Strains important in fish and meat spoilage. Psychrotrophs.

Flavobacterium. Rods with parallel sides (0.5×3 μm); nonmotile; colonies colored; some species psychrotrophs. Cause spoilage of milk, meat, and other protein foods. Species: *Flavobacterium aquatile*.

Alcaligenes. Rods or coccobacilli (0.5×1 μm); motile; present in water, soil, or fecal material; mesophiles. Cause spoilage of protein-rich foods. Species: *Alcaligenes faecalis*.

Brucella. Coccobacilli (0.5×1.0 μm); mostly single; nonmotile. Different species cause disease in animals, including cattle, pigs, and sheep. They are also human pathogens and have been implicated in foodborne brucellosis. *Brucella abortus* causes abortion in cows.

Psychrobacter. The genus was created in 1986 and contains one species — *Psychrobacter immobilis*. Coccobacilli (1×1.5 μm) and nonmotile. Can grow at 5°C or below, show optimum growth at 20°C, and unable to grow at 35°C. Found in fish, meat, and poultry products.

2. Gram-Negative Facultative Anaerobes

Citrobacter. Straight rods (1×4 μm); single or in pairs; usually motile; mesophiles. Found in the intestinal contents of humans, animals, and birds, and in the environment. Included in the coliform group as an indicator of sanitation. Important species: *Citrobacter freundii*.

Escherichia. Straight rods (1×4 μm); motile or nonmotile; mesophiles. Found in the intestinal contents of humans, warm-blooded animals, and birds. Many strains nonpathogenic, but some strains pathogenic to humans and animals and involved in foodborne diseases. Used as an indicator of sanitation (theoretically nonpathogenic strains) in coliform and fecal coliform groups. Important species: *Escherichia coli*.

Enterobacter. Straight rods (1×2 μm); motile; mesophiles. Found in the intestinal contents of humans, animals, birds, and in the environment. Included in the coliform group as an indicator of sanitation. Important species: *Enterobacter aerogenes*.

Edwardsiella. Small rods (1 × 2 μm); motile. Found in the intestines of cold-blooded animals and in fresh water. Can be pathogenic to humans, but involvement in foodborne disease not shown.

Erwinia. Small rods (1 × 2 μm); occur in pairs or short chains; motile; facultative anaerobes; optimum growth at 30°C. Many are plant pathogens and cause spoilage of plant products. Species: *Erwinia amylovora*.

Hafnia. Small rods (1 × 2 μ); motile; mesophiles. Found in intestinal contents of humans, animals, and birds, and in the environment. Associated with food spoilage. Species: *Hafnia alvei*.

Klebsiella. Medium rods (1 × 4 μm); occur singly or in pairs; motile; capsulated; mesophiles. Found in the intestinal contents of humans, animals, and birds; soil; water; and grains. Included in the coliform group as an indicator of sanitation. Important species: *Klebsiella pneumoniae*.

Morganella. Small rods (0.5 × 1 μm), motile, mesophiles. Found in the intestinal contents of humans and animals. Can be pathogenic but has not been implicated in foodborne disease. Species: *Morganella morganii*.

Proteus. Straight, small rods (0.5 × 1.5 μm); highly motile; form swarm on agar media; some grow at low temperature. Occur in the intestinal contents of humans and animals and the environment. Many involved in food spoilage. Species: *Proteus vulgaris*.

Salmonella. Medium rods (1 × 4 μm); usually motile; mesophiles. There are over 2000 serovars and all are regarded as human pathogens. Found in the intestinal contents of humans, animals, birds, and insects. Major cause of foodborne diseases. Species: *Salmonella enterica* ssp. *enterica.* (See Chapter 25 for the new naming system.)

Shigella. Medium rods; nonmotile; mesophiles. Found in the intestine of humans and primates. Associated with foodborne diseases. Species: *Shigella dysenteriae*.

Serratia. Small rods (0.5 × 1.5 μm); motile; colonies white, pink, or red; some grow at refrigerated temperature. Occur in the environment. Cause food spoilage. Species: *Serratia liquefaciens*.

Yersinia. Small rods (0.5 × 1 μm); motile or nonmotile; can grow at 1°C. Present in the intestinal contents of animals. *Yersinia enterocolitica* has been involved in foodborne disease outbreaks.

Vibrio. Curved rods (0.5 × 1.0 μm); motile; mesophiles. Found in freshwater and marine environments. Some species need NaCl for growth. Several species are pathogens and have been involved in foodborne disease (*Vibrio cholerae, Vib. parahaemolyticus,* and *Vib. vulnificus*), whereas others can cause food spoilage (*Vib. alginolyticus*).

Aeromonas. Small rods (0.5 × 1.0 μm); occur singly or in pairs; motile; psychrotrophs. Found in a water environment. *Aeromonas hydrophila* has been suspected as a potential foodborne pathogen.

Plesiomonas. Small rods (0.5 × 1.0 μm), motile. Found in fish and aquatic animals. *Plesiomonas shigelloides* has been suspected as a potential foodborne pathogen.

3. Rickettsias

Coxiella. Gram-negative; nonmotile; very small cells (0.2 × 0.5 μm); grow on host cells. Relatively resistant to high temperature (killed by pasteurization). *Coxiella burnetii* causes infection in cattle and has been implicated with Q fever in humans (especially on consuming unpasteurized milk).

4. Gram-Positive Cocci

Micrococcus. Spherical cells (0.2 to 2 μm); occur in pairs, tetrads, or clusters; aerobes; nonmotile; some species produce yellow colonies; mesophiles, resistant to low heat. Found in mammalian skin. Can cause spoilage. Species: *Micrococcus luteus*.

Staphylococcus. Spherical cells (0.5 to 1 μm); occur singly, in pairs, or clusters; nonmotile; mesophiles; facultative anaerobes; grow in 10% NaCl. *Staphylococcus aureus* strains are frequently involved in foodborne diseases. *Sta. carnosus* is used for processing some fermented sausages. Main habitat is skin of humans, animals, and birds.

Streptococcus. Spherical or ovoid (1 μm); occur in pairs or chains; nonmotile; facultative anaerobes; mesophiles. *Streptococcus pyogenes* is pathogenic and has been implicated in foodborne diseases; present as commensals in human respiratory tract. *Str. thermophilus* is used in dairy fermentation; can be present in raw milk; can grow at 50°C.

Enterococcus. Spheroid cells (1 μm); occur in pairs or chains; nonmotile; facultative anaerobes; some strains survive low heat (pasteurization); mesophiles. Normal habitat is the intestinal contents of humans, animals, and birds, and the environment. Can establish on equipment surfaces. Used as an indicator of sanitation. Important in food spoilage. Species: *Enterococcus faecalis*.

Lactococcus. Ovoid elongated cells (0.5 to 1.0 μm); occur in pairs or short chains; nonmotile; facultative anaerobes; mesophiles, but can grow at 10°C; produce lactic acid. Used to produce many bioprocessed foods, especially fermented dairy foods. Species: *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*; present in raw milk and plants and several strains produce bacteriocins, some with a relatively wide host range against Gram-positive bacteria and have potential as food biopreservatives.

Leuconostoc. Spherical or lenticular cells; occur in pairs or chains; nonmotile; facultative anaerobes; heterolactic fermentators; mesophiles, but some species and strains can grow at or below 3°C. Some are used in food fermentation. Psychrotrophic strains are associated with spoilage (gas formation) of vacuum-packaged refrigerated foods. Found in plants, meat, and milk. Species: *Leuconostoc mesenteroides* subsp. *mesenteroides, Leu. lactis, Leu. carnosum. Leu. mesenteroides* subsp. *dextranicum* produces dextran while growing in sucrose. Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and these have potential as food biopreservatives.

Pediococcus. Spherical cells (1 μm); form tetrads; mostly present in pairs; nonmotile; facultative anaerobes; homolactic fermentators; mesophiles, but some can grow at 50°C; some survive pasteurization. Some species and strains are used in food fermentation. Some can cause spoilage of alcoholic beverages. Found in vegetative materials and in some food products. Species: *Pediococcus acidilactici* and *Ped. pentosaceus.* Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and they can be used as food biopreservatives.

Sarcina. Large, spherical cells (1 to 2 μm); occur in packets of eight or more; nonmotile; produce acid and gas from carbohydrates; facultative anaerobes. Present in soil, plant products, and animal feces. Can be involved in spoilage of foods of plant origin. Species: *Sarcina maxima*.

5. Gram-Positive, Endospore-Forming Rods

Bacillus. Rod-shaped, straight cells; vary widely in size (small, medium, or large; 0.5–1 × 2–10 μm) and shape (thick or thin); single or in chains; motile or nonmotile; mesophiles or psychrotrophic; aerobes or facultative anaerobes; all form endospores that are spherical or oval and large or small (one per cell), spores are highly heat resistant. Includes many species, some of which are important in foods, because they can cause foodborne disease (*Bacillus cereus*) and food spoilage, especially in canned products (*Bac. coagulans, Bac. stearothermophilus*). Enzymes of some species and strains are used in food bioprocessing (*Bac. subtilis*). Present in soil, dust, and plant products (especially spices). Many species and strains can produce extracellular enzymes that hydrolyze carbohydrates, proteins, and lipids.

Sporolactobacillus. Slender, medium-sized rods (1 × 4 μm); motile; microaerophilic; homolactic fermentors; form endospores (spore formation is rare in most media), but the spores are less heat resistant than *Bacillus* spores. Found in chicken feed and soil. Importance in food is not clearly known. Species: *Sporolactobacillus inulinus.*

Clostridium. Rod-shaped cells that vary widely in size and shape; motile or nonmotile; anaerobes (some species extremely sensitive to oxygen); mesophiles or psychrotrophic; form endospores (oval or spherical) usually at one end of the cell, some species sporulate poorly, spores are heat resistant. Found in soil, marine sediments, sewage, decaying vegetation, and animal and plant products. Some are pathogens and important in food (*Clostridium botulinum, Clo. perfringens*) and others are important in food spoilage (*Clo. tyrobutyricum, Clo. saccharolyticum, Clo. laramie*). Some species are used as sources of enzymes to hydrolyze carbohydrates and proteins in food processing.

6. Gram-Negative, Endospore-Forming Rods

Desulfotomaculum. One species important in food is *Delsufatomaculum nigrificans*. The medium-sized cells are rod shaped, motile, thermophilic, strictly anaerobes, and produce H_2S. Endospores are oval and resistant to heat. Found in soil. Cause spoilage of canned food.

7. Gram-Positive, Nonsporulating Regular Rods

Lactobacillus. Rod-shaped cells that vary widely in shape and size, some are very long whereas others are coccobacilli, appear in single or in small and large chains; facultative anaerobes; most species are nonmotile; mesophiles (but some are psychrotrophs); can be homo- or heterolactic fermentors. Found in plant sources, milk, meat, and feces. Many are used in food bioprocessing (*Lactobacillus delbrueckii* subsp. *bulgaricus, Lab. helveticus, Lab. plantarum*) and some are used as probiotics (*Lab. acidophilus, Lab. reuteri, Lab. casei* subsp. *casei*). Some species can grow at low temperatures in products stored at refrigerated temperature (*Lab. sake, Lab. curvatus*). Several strains produce bacteriocins, of which some having a wide spectrum can be used as food biopreservatives.

Carnobacterium. Similar in many characteristics to lactobacilli cells; found in meat and fish; facultative anaerobes; heterofermentative; nonmotile; can grow in foods,

especially in meat products; stored at refrigerated temperature. Some strains produce bacteriocins. Species: *Carnobacterium piscicola*.

Brochothrix. Similar in many characteristics to lactobacilli; facultative anaerobes; homofermentative; nonmotile; found in meat. Can grow in refrigerated vacuum-packaged meat and meat products. Species: *Brochothrix thermosphacta*.

Listeria. Short rods (0.5×1 μm); occur singly or in short chains; motile; facultative anaerobes; can grow at 1°C; cells killed by pasteurization. The species are widely distributed in the environment and have been isolated from different types of foods. Some *Listeria monocytogenes* strains are important foodborne pathogens.

8. Gram-Positive, Nonsporeforming Irregular Rods

Corynebacterium. Slightly curved rods; some cells stain unevenly; facultative anaerobes; nonmotile; mesophiles; found in the environment, plants, and animals. Some species cause food spoilage. *Corynebacterium glutamicum* is used to produce glutamic acid.

Brevibacterium. Cells can change from rod to coccoid shape; aerobes; nonmotile; mesophiles. Two species, *Brevibacterium linens* and *Bre. casei*, have been implicated in the development of the aroma in several cheese varieties (surface ripened), because of the production of sulfur compounds (such as methanethiol). In other protein-rich products, they can cause spoilage (in fish). They are found in different cheeses and raw milk.

Propionibacterium. Pleomorphic rods (0.5×2 μm); can be coccoid, bifid, or branched; present singly or in short chains; V and Y configuration and in clumps with Chinese-character-like arrangement; nonmotile; anaerobes; mesophiles. Dairy propionibacteria are used in food fermentation (*Propionibacterium freudenreichii* in Swiss cheese). Produce proline and propionic acid. Found in raw milk, Swiss cheese, and silage.

Bifidobacterium. Rods of various shapes; present singly or in chains; arranged in V or star-like shape; nonmotile; mesophiles; anaerobes. Metabolize carbohydrates to lactate and acetate. Found in colons of humans, animals, and birds. Some species are used in probiotics (*Bifidobacterium bifidum, Bif. infantis, Bif. adolescentis*).

9. Some New Genera

Based on nucleic acid homology studies, several new genera have been created from the existing genera. Some that are important in food microbiology are listed here. Most of their characteristics are similar to those of the species in the respective old genera. Examples include *Tetragenococcus* (from *Pediococcus*), *Vagococcus* (from N-group *Streptococcus*), *Weissella* and *Oenococcus* (both from *Leuconostoc*), *Kocuria* (from *Micrococcus*), *Shewanella* (from *Pseudomonas*), and *Alicyclobacillus* (from *Bacillus*).

VI. IMPORTANT BACTERIAL GROUPS IN FOODS

Among the microorganisms found in foods, bacteria constitute major important groups.[11] This is not only because many different species can be present in foods,

but also becuase of their rapid growth rate, ability to utilize food nutrients, and ability to grow under a wide range of temperatures, aerobiosis, pH, and water activity, as well as to better survive adverse situations, such as survival of spores at high temperature. For convenience, bacteria important in foods have been arbitrarily divided into several groups on the basis of similarities in certain characteristics. This grouping does not have any taxonomic significance. Some of these groups and their importance in foods are listed here.

A. Lactic Acid Bacteria

They are bacteria that produce relatively large quantities of lactic acid from carbohydrates. Species mainly from genera *Lactococcus, Leuconostoc, Pediococcus, Lactobacillus*, and *Streptococcus thermophilus* are included in this group.

B. Acetic Acid Bacteria

They are bacteria that produce acetic acid, such as *Acetobacter aceti*.

C. Propionic Acid Bacteria

They are bacteria that produce propionic acid and are used in dairy fermentation. Species such as *Propionibacterium freudenreichii* are included in this group.

D. Butyric Acid Bacteria

They are bacteria that produce butyric acid in relatively large amounts. Some *Clostridium* spp. such as *Clostridium butyricum* are included in this group.

E. Proteolytic Bacteria

They are bacteria that can hydrolyze proteins because they produce extracellular proteinases. Species in genera *Micrococcus, Staphylococcus, Bacillus, Clostridium, Pseudomonas, Alteromonas, Flavobacterium, Alcaligenes*, some in *Enterobacteriaceae*, and *Brevibacterium* are included in this group.

F. Lipolytic Bacteria

They are bacteria that are able to hydrolyze triglycerides because they produce extracellular lipases. Species in genera *Micrococcus, Staphylococcus, Pseudomonas, Alteromonas,* and *Flavobacterium* are included in this group.

G. Saccharolytic Bacteria

They are bacteria that are able to hydrolyze complex carbohydrates. Species in genera *Bacillus, Clostridium, Aeromonas, Pseudomonas,* and *Enterobacter* are included in this group.

H. Thermophilic Bacteria

They are bacteria that are able to grow at 50°C and above. Species from genera *Bacillus, Clostridium, Pediococcus, Streptococcus,* and *Lactobacillus* are included in this group.

I. Psychrotrophic Bacteria

They are bacteria that are able to grow at refrigerated temperature (≤5°C). Some species from *Pseudomonas, Alteromonas, Alcaligenes, Flavobacterium, Serratia, Bacillus, Clostridium, Lactobacillus, Leuconostoc, Carnobacterium, Brochothrix, Listeria, Yersinia*, and *Aeromonas* are included in this group.

J. Thermoduric Bacteria

They are bacteria that are able to survive pasteurization temperature treatment. Some species from *Micrococcus, Enterococcus, Lactobacillus*, *Pediococcus, Bacillus* (spores), and *Clostridium* (spores) are included in this group.

K. Halotolerant Bacteria

They are bacteria that are able to survive high salt concentrations (≥10%). Some species from *Bacillus, Micrococcus, Staphylococcus, Pediococcus, Vibrio,* and *Corynebacterium* are included in this group.

L. Aciduric Bacteria

They are bacteria that are able to survive at low pH (<4.0). Some species from *Lactobacillus, Pediococcus, Lactococcus, Enterococcus,* and *Streptococcus* are included in this group.

M. Osmophilic Bacteria

They are bacteria that can grow at a relatively higher osmotic environment than that needed for other bacteria. Some species from genera *Staphylococcus, Leuconostoc,* and *Lactobacillus* are included in this group. They are much less osmophilic than yeasts and molds.

N. Gas-Producing Bacteria

They are bacteria that produce gas (CO_2, H_2, H_2S) during metabolism of nutrients. Species from genera *Leuconostoc, Lactobacillus, Propionibacterium, Escherichia, Enterobacter, Clostridium,* and *Desulfotomaculum* are included in this group.

O. Slime Producers

They are bacteria that produce slime because they synthesise polysaccharides. Some species or strains from *Xanthomonas, Leuconostoc, Alcaligenes, Enterobacter, Lactococcus,* and *Lactobacillus* are included in this group.

P. Spore Formers

They are bacteria having the ability to produce spores. Species from *Bacillus, Clostridium*, and *Desulfotomaculum* are included in this group. They are further divided into aerobic sporeformers, anaerobic sporeformers, flat sour sporeformers, thermophilic sporeformers, and sulfide-producing sporeformers.

Q. Aerobes

They are bacteria that require oxygen for growth and multiplication. Species from *Pseudomonas, Bacillus,* and *Flavobacterium* are included in this group.

R. Anaerobes

They are bacteria that cannot grow in the presence of oxygen. Species from *Clostridium* are included in this group.

S. Facultative Anaerobes

They are bacteria that are able to grow in both the presence and absence of oxygen. *Lactobacillus, Pediococcus, Leuconostoc*, enteric pathogens, and some species of *Bacillus, Serratia,* and coliforms are included in this group.

T. Coliforms

Species from *Escherichia, Enterobacter, Citrobacter,* and *Klebsiella* are included in this group. They are used as an index of sanitation.

U. Fecal Coliforms

Mainly *Escherichia coli* is included in this group. They are also used as an index of sanitation.

V. Enteric Pathogens

Pathogenic *Salmonella, Shigella, Campylobacter, Yersinia, Escherichia, Vibrio, Listeria,* hepatitis A, and others that can cause gastrointestinal infection are included in this group.

Because of the importance of these bacterial groups in food, many laboratory methods are designed to detect a specific group instead of a specific genus or species. Similarly, control methods are sometimes designed to destroy or prevent growth of a specific group.

VII. CONCLUSION

Two major aspects have been discussed in this chapter: the nomenclature system of bacteria, which is rapidly changing because of the development of new molecular biology techniques, and the morphological and physiological characteristics of the microorganisms important in food. A food microbiologist should be knowledgeable about these facets. Chapter 3 discusses the sources of these microorganisms in food.

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QUESTIONS

1. List five methods that are used in the classification of bacteria. Why are nucleotide sequences in 16S rRNA used as an important technique in the classification?
2. Explain the following terms and give one example for each in relation to bacterial nomenclature: (a) family, (b) genus, (c) species, (d) subspecies, and (e) biovar

(use scientific method in the examples). Give the plural of *Lactobacillus, Staphylococcus, Enterococcus, Leuconostoc, Listeria*, and *Salmonella*.

3. List the general differences in the morphology of yeasts, molds, bacteria, and bacteriophages important in food.
4. List the differences in the chemical nature and function of cell wall structures between Gram-positive and Gram-negative bacteria. How does this help determine Gram characteristics of an unknown bacterial isolate?
5. List four species of molds and two species of yeasts most important in food.
6. Discuss the importance of bacteriophages in food. Briefly explain how prions are different from the bacteriophages.
7. List two genera from each of the following groups: (a) Gram-negative aerobic rods, (b) Gram-negative facultative anaerobic rods, (c) Gram-positive cocci, (d) Gram-positive endospore-forming rods, (e) Gram-positive nonsporulating rods.
8. Briefly mention the common characteristics used to group bacterial genera or species in the following groups and give one example of genus for each: (a) lactic acid bacteria, (b) coliforms, (c) proteolytic bacteria, (d) psychrotrophic bacteria, and (e) enteric pathogens.

CHAPTER 3

Sources of Microorganisms in Foods

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I. INTRODUCTION

The internal tissues of healthy plants (fruits and vegetables) and animals (meat) are essentially sterile. Yet raw and processed (except sterile) foods contain different types of molds, yeasts, bacteria, and viruses. Microorganisms get into foods from both natural (including internal) sources and from external sources to which a food comes into contact from the time of production until the time of consumption. Natural sources for foods of plant origin include the surfaces of fruits, vegetables, and grains, and the pores in some tubers (e.g., radish and onion). Natural sources for foods of animal origin include skin, hair, feathers, gastrointestinal tract, urinogenital tract, respiratory tract, and milk ducts (teat canal) in udders of milk animals.

Natural microflora exist in ecological balance with their hosts, and their types and levels vary greatly with the type of plants and animals as well as their geographical locations and environmental conditions. Besides natural microorganisms, a food can be contaminated with different types of microorganisms coming from outside sources such as air, soil, sewage, water, feeds, humans, food ingredients, equipment, packages, and insects. Microbial types and their levels from these sources getting into foods vary widely and depend on the degree of sanitation used during the handling of foods.

An understanding of the sources of microorganisms in food is important to develop methods to control access of some microorganisms in the food, develop processing methods to kill them in food, and determine the microbiological quality of food, as well as set up microbiological standards and specifications of foods and food ingredients. The predominant types that can get into food from each of these sources and methods to reduce the levels of microorganisms are briefly discussed here.

II. PREDOMINANT MICROORGANISMS IN DIFFERENT SOURCES

A. Plants (Fruits and Vegetables)

The inside tissue of foods from plant sources are essentially sterile, except for a few porous vegetables (e.g., radishes and onions) and leafy vegetables (e.g., cabbage and Brussels sprouts).[1–5] Some plants produce natural antimicrobial metabolites that can limit the presence of microorganisms. Fruits and vegetables harbor microorganisms on the surface; their type and level vary with soil condition, type of fertilizers and water used, and air quality. Molds, yeasts, lactic acid bacteria, and bacteria from genera *Pseudomonas, Alcaligenes, Micrococcus, Erwinia, Bacillus, Clostridium*, and *Enterobacter* can be expected from this source. Pathogens, especially of enteric types, can be present if the soil is contaminated with untreated sewage. Diseases of the plants, damage of the surface (before, during, and after harvest), long delay between harvesting and washing, and unfavorable storage and transport conditions after harvesting and before processing can greatly increase microbial numbers as well as predominant types. Improper storage conditions following processing can also increase their numbers.

Proper methods used during growing (such as use of treated sewage or other types of fertilizers), damage reduction during harvesting, quick washing with good-quality water to remove soil and dirt, and storage at low temperature before and after processing can be used to reduce microbial load in foods of plant origin.

B. Animals, Birds, Fish, and Shellfish

Food animals and birds normally carry many types of indigenous microorganisms in the digestive, respiratory, and urinogenital tracts, the teat canal in the udder, as well as in the skin, hooves, hair, and feathers. Their numbers, depending on the specific organ, can be very high (large intestinal contents can have as high as 10^{10} bacteria/g). Many, as carriers, can harbor pathogens such as *Salmonella* serovars,

pathogenic *Escherichia coli, Campylobacter jejuni, Yersinia enterocolitica*, and *Listeria monocytogenes* without showing symptoms. Laying birds have been suspected of asymptomatically carrying *Salmonella* Enteritidis in the ovaries and contaminating the yolk during ovulation. Disease situations, such as mastitis in cows and intestinal, respiratory, and uterine infections, as well as injury can change the ecology of normal microflora. Similarly, poor husbandry resulting in fecal contamination on the body surface (skin, hair, feathers, and udder) and supplying contaminated water and feed (e.g., contaminated with salmonellae) can also change their normal microbial flora.

Fish and shellfish also carry normal microflora in the scales, skin, and digestive tracts. Water quality, feeding habits, and diseases can change the normal microbial types and level. Pathogens such as *Vibrio parahaemolyticus, Vib. vulnificus,* and *Vib. cholerae* are of major concern from these sources. Many spoilage and pathogenic microorganisms can get into foods of animal origin (milk, egg, meat, and fishery products) during production and processing. Milk can be contaminated with fecal materials on the udder surface, egg shells with fecal material during laying, meat with the intestinal contents during slaughtering, and fish with intestinal contents during processing. Because of its specific nature, contamination of foods of animal sources with fecal materials is viewed with concern (possible presence of enteric pathogens).

In addition to enteric pathogens from fecal materials, meat from food animals and birds can be contaminated with several spoilage and pathogenic microorganisms from skin, hair, and feathers, namely *Staphylococcus aureus, Micrococcus* spp., *Propionibacterium* spp., *Corynebacterium* spp., and molds and yeasts.

Prevention of food contamination from these sources needs the use of effective husbandry of live animals and birds, which includes good housing and supply of uncontaminated feed and water. Also, testing animals and birds for pathogens and culling the carriers are important in reducing the incidence of pathogenic microorganisms in foods. Thorough cleansing using good-quality water for washing carcasses (preferably with acceptable antimicrobial agents); hair removal; feather removal; careful removal of digestive, urinogenital, and respiratory organs without contaminating tissues; removal of contaminated parts; and proper sanitation during the entire processing stage are necessary during slaughter to keep microbial quantity and quality at desirable levels. Proper cleaning of the udder before milking, cooling milk immediately after milking, processing as soon as possible, and sanitization at all stages are important to keep microbial levels low in milk. Eggs should be collected soon after laying and washed and stored as per recommended procedures.

Fish and marine products should be harvested from unpolluted and recommended water. Proper sanitation should be used during processing. They should be stored properly to prevent further contamination and microbial growth. Ice to be used for storage should be produced from potable water.

C. Air

Microorganisms are present in dust and moisture droplets in the air. They do not grow in dust, but are transient and variable, depending on the environment. Their

level is controlled by the degree of humidity, size and level of dust particles, temperature and air velocity, and resistance of microorganisms to drying. Generally, dry air with low dust content and higher temperature has a low microbial level. Spores of *Bacillus* spp., *Clostridium* spp., and molds, and cells of some Gram-positive bacteria (e.g., *Micrococcus* spp. and *Sarcina* spp.), as well as yeasts, can be predominantly present in air. If the surroundings contain a source of pathogens (e.g., animal and poultry farms or a sewage-treatment plant), different types of bacteria, including pathogens and viruses (including bacteriophages), can be transmitted via the air.

Microbial contamination of food from the air can be reduced by removing the potential sources, controlling dust particles in the air (using filtered air), using positive air pressure, reducing humidity level, and installing UV light.

D. Soil

Soil, especially the type used to grow agricultural produce and raise animals and birds, contains several varieties of microorganisms. Because microorganisms can multiply in soil, their numbers can be very high (billions/g). Many types of molds, yeasts, and bacterial genera (e.g., *Enterobacter, Pseudomonas, Proteus, Micrococcus, Enterococcus, Bacillus,* and *Clostridium*) can enter foods from the soil. Soil contaminated with fecal materials can be the source of enteric pathogenic bacteria and viruses in food. Sediments where fish and marine foods are harvested can also be a source of microorganisms, including pathogens, in those foods. Different types of parasites can also get in food from soil. Removal of soil (and sediments) by washing and avoiding soil contamination can reduce microorganisms in foods from this source.

E. Sewage

Sewage, especially when used as fertilizer in crops, can contaminate food with microorganisms, the most significant of which are different enteropathogenic bacteria and viruses. This can be a major concern with organically grown food and many imported fruits and vegetables, in which untreated sewage and manure might be used as fertilizer. Pathogenic parasites can also get in food from sewage.

To reduce incidence of microbial contamination of foods from sewage, it is better not to use sewage as fertilizer. If used, it should be efficiently treated to kill the pathogens. Also, effective washing of foods following harvesting is important.

F. Water

Water is used to produce, process, and, under certain conditions, store foods. It is used for irrigation of crops, drinking by food animals and birds, raising fishery and marine products, washing foods, processing (pasteurization, canning, and cooling of heated foods) and storage of foods (e.g., fish on ice), washing and sanitation of equipment, and processing and transportation facilities. Water is also used as an ingredient in many processed foods. Thus, water quality can greatly influence micro-

bial quality of foods. Contamination of foods with pathogenic bacteria, viruses, and parasites from water has been recorded.

Wastewater can be recycled for irrigation. However, chlorine-treated potable water (drinking water) should be used in processing, washing, sanitation, and as an ingredient. Although potable water does not contain coliforms and pathogens (mainly enteric types), it can contain other bacteria capable of causing food spoilage, such as *Pseudomonas, Alcaligenes*, and *Flavobacterium.* Improperly treated water can contain pathogenic and spoilage microorganisms. To overcome the problems, many food processors use water, especially as an ingredient, that has a higher microbial quality than that of potable water.

G. Humans

Between production and consumption, foods come in contact with different people handling the foods. They include not only people working in farms and food-processing plants, but also those handling foods at restaurants, catering services, retail stores, and at home. Human carriers have been the source of pathogenic microorganisms in foods that later caused foodborne diseases, especially with ready-to-eat foods. Improperly cleaned hands, lack of aesthetic sense and personal hygiene, and dirty clothes and hair can be major sources of microbial contamination in foods. The presence of minor cuts and infection in hands and face and mild generalized diseases (e.g., flu, strep throat, or hepatitis A in an early stage) can amplify the situation. In addition to spoilage bacteria, pathogens such as *Sta. aureus, Salmonella* serovars, *Shigella* spp., pathogenic *Esc. coli*, and hepatitis A can be introduced into foods from human sources.

Proper training of personnel in personal hygiene, regular checking of health, and maintaining efficient sanitary and aesthetic standards are necessary to reduce contamination from this source.

H. Food Ingredients

In prepared or fabricated foods, many ingredients or additives are included in different quantities. Many of these ingredients can be the source of both spoilage and pathogenic microorganisms. Various spices generally have very high populations of mold and bacterial spores. Starch, sugar, and flour might have spores of thermophilic bacteria. Pathogens have been isolated from dried coconut, egg, and chocolate.

The ingredients should be produced under sanitary conditions and given antimicrobial treatments. In addition, setting up acceptable microbial specifications for the ingredients will be important in reducing microorganisms in food from this source.

I. Equipment

A wide variety of equipment is used in harvesting, slaughtering, transporting, processing, and storing foods. Many types of microorganisms from air, raw foods, water, and personnel can get into the equipment and contaminate foods. Depending on the environment (moisture, nutrients, and temperature) and time, microorganisms can

multiply and, even from a low initial population, reach a high level and contaminate large volumes of foods. Also, when processing equipment is used continuously for a long period of time, microorganisms initially present can multiply and act as a continuous source of contamination in the product produced subsequently. In some equipment, small parts, inaccessible sections, and certain materials might not be efficiently cleaned and sanitized. These dead spots can serve as sources of both pathogenic and spoilage microorganisms in food. Small equipment, such as cutting boards, knives, spoons, and similar articles, because of improper cleaning, can be sources of cross contamination. *Salmonella, Listeria, Escherichia, Enterococcus, Micrococcus, Pseudomonas, Lactobacillus, Leuconostoc, Clostridium, Bacillus* spp., and yeasts and molds can get in food from equipment (see Chapter 21).

Proper cleaning and sanitation of equipment at prescribed intervals are important to reduce microbial levels in food. In addition, developing means to prevent or reduce contamination from air, water, personnel, and insects is important. Finally, in designing the equipment, potential microbiological problems need to be considered.

J. Miscellaneous

Foods might be contaminated with microorganisms from several other sources, namely packaging and wrapping materials, containers, flies, vermins, birds, house pets, and rodents. Many types of packaging materials are used in food. Because they are used in products ready for consumption and in some cases without further heating, proper microbiological standards (or specifications) for packaging materials are necessary. Any failure to produce microbiologically acceptable products can reduce the quality of food. Flies, vermins, birds, and rodents in food processing and food preparation and storage facilities should be viewed with concern as they can carry pathogenic microorganisms. House pets can also harbor pathogens; proper care should be taken not to contaminate food from this source.

III. CONCLUSION

Microorganisms enter foods from both internal and external sources. Their levels and types depend on the care used during production, processing, and storage of foods. Recontaminations of heat-processed foods with pathogens have been associated with many foodborne disease outbreaks. Proper sanitation at every stage helps reduce the microbial level normally expected in a food. Normal microbiological quality of foods is discussed in Chapter 4.

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QUESTIONS

1. Briefly discuss how an understanding of the microbial sources in food can be helpful to a food microbiologist.
2. List five major sources of foodborne pathogens in foods and indicate the measures that should be implemented to reduce their incidence in foods.
3. A *Salmonella* outbreak from the consumption of a national brand of ice cream, involving more than 50,000 consumers, was found to be related to contamination of heat-treated ice cream mix with raw liquid egg containing the pathogen. Both products were transported by the same truck at different times. Briefly indicate how this could have been avoided.
4. A batch of turkey rolls (10 lb — about 4.5 Kg — each) were cooked to 165°F internal temperature in bags, opened, sliced, vacuum-packaged, and stored at 40°F. The product was expected to have a refrigerated shelf life of 50 days. However, after 40 days, the packages contained gas and ca. 10^7 bacterial cells/g of meat. The bacterial species involved in the spoilage was found to be *Leuconostoc carnosum,* which is killed at 165°F. What could be the sources of the bacterial species in this cooked product?
5. Consumption of vacuum-packaged refrigerated hotdogs has been frequently incriminated with listeriosis, although the internal temperature used for cooking the raw products (160°F) is high enough to kill the pathogen. Discuss the possible sources of the pathogen in the cooked products.
6. A child consumed watermelon in a restaurant and was thereafter infected with *Esc. coli* O157:H7. Investigations revealed that the knife used to slice the melon was also used to cut raw beef (and then washed). What could have been done to prevent the situation?

CHAPTER 4

Normal Microbiological Quality of Foods and its Significance

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I. INTRODUCTION

Although many types (genera, species, strains) of microorganisms are present in nature, under normal conditions a food generally harbors only a few types. These types include those that are naturally present in raw foods (which provide the ecological niche) and those that enter from outside sources to which the foods are exposed from the time of production until consumption. The relative numbers (population level) of a specific type of microorganism initially present (without growth) in a food depend on the intrinsic and extrinsic conditions to which the food is exposed. If growth occurs, the predominant types will be the ones for which the

optimum growth condition is present in the food. This aspect is discussed in Chapter 6. The objective of this chapter is to develop an understanding of the microbial types (and their levels where possible) that can be expected under normal conditions in different food groups. It has to be recognized that microbial load in a food results from initial contamination from different sources and growth of the contaminants before testing; normally, it is difficult to separate the two.

II. RAW AND READY-TO-EAT MEAT PRODUCTS

Following slaughter and dressing, the carcasses of animals and birds contain many types of microorganisms, predominantly bacteria, coming from the skin, hair, feathers, gastrointestinal tract, etc.; the environment of the feedlot and pasture (feed, water, soil, and manure); and the environment at the slaughtering facilities (equipment, air, water, and humans). Normally, carcasses contain an average of 10^{1-3} bacterial cells/in.^{2}. Different enteric pathogens, *Salmonella* serovars, *Yersinia enterocolitica, Campylobacter jejuni, Escherichia coli, Clostridium perfringens*, and *Staphylococcus aureus*, both from animals or birds and humans, can be present, but normally at a low level. Carcasses of birds, as compared with those of animals, generally have a higher incidence of *Salmonella* contamination coming from fecal matter.[1–4]

Following boning, chilled raw meat and ground meat contain microorganisms coming from the carcasses as well as from different equipment used during processing, personnel, air, and water. Some of the equipment used can be important sources of microorganisms, such as conveyors, grinders, slicers, and similar types that can be difficult to clean. Chilled meat has mesophiles, such as *Micrococcus, Enterococcus, Staphylococcus, Bacillus, Clostridium, Lactobacillus*, coliforms, and other *Enterobacteriaceae*, including enteric pathogens. However, because the meats are stored at low temperature (–1 to 5°C), the psychrotrophs constitute major problems. The predominant psychrotrophs in raw meats are some lactobacilli and leuconostocs, *Brochothrix thermosphacta, Clo. laramie*, some coliforms, *Serratia, Pseudomonas, Alteromonas, Achromobacter, Alcaligenes, Acinetobacter, Morexella, Aeromonas,* and *Proteus*. Psychrotrophic pathogens include *Listeria monocytogenes* and *Yer. enterocolitica*. The microbial load of fresh meat varies greatly, with bacteria predominating. Ground meat can have 10^{4-5} microorganisms/g; *Salmonella* can be present at ca. 1 cell/25 g. As indicated before, the frequency of the presence of *Salmonella* is higher in chicken than in red meats. If the products are kept under aerobic conditions, psychrotrophic aerobes will grow rapidly, especially Gram-negative rods, such as *Pseudomonas, Alteromonas, Proteus,* and *Alcaligenes*, as well as yeasts. Under anaerobic packaging, growth of psychrotrophic facultative anaerobes and anaerobes (e.g., *Lactobacillus, Leuconostoc, Brochothrix, Serratia*, some coliforms, and *Clostridium*) predominates. The pH of the meat (which is low in beef, ca. 5.6, but high in birds, ca. 6.0), high protein content, and low carbohydrate level, along with the environment, determine which types predominate during storage.

Low-heat-processed red meat and poultry products include perishable cured or uncured products that have been subjected to heat treatment to ca. 160°F (70°C) , packaged aerobically or anaerobically, and stored at refrigerated temperature. They include products such as franks, bologna, lunchmeats, and hams. The products, especially those packaged anaerobically and cured, are expected to have a long storage life (50 d or more). The microbial sources before heat treatment include the raw meat, ingredients used in formulation, processing equipment, air, and personnel. Heat treatment, especially at an internal temperature of 160°F or higher, kills most microorganisms, except some thermodurics, which include *Micrococcus*, some *Enterococcus*, and maybe some *Lactobacillus* and spores of *Bacillus* and *Clostridium*. The microbial level can be 10^{1-2}/ g. Following heating, the products, some of which are further processed (such as removing casing or slicing) come in contact with equipment, personnel, air, and water before final packaging. Different types of bacteria, yeasts, and molds, including pathogens, can enter these products, depending on the conditions of the processing plants. Although the initial bacterial level normally does not exceed 10^2/g, some of them can be psychrotrophic facultative anaerobic and anaerobic bacteria (*Lactobacillus, Leuconostoc*, some coliforms, *Serratia, Listeria, Clostridium* spp.). During extended storage in vacuum or modified-air packages, even from a low initial level, bacterial population can rise and adversely affect the safety and shelf life of products. This is aggravated by fluctuation in storage temperature and in products having low fat, high pH, and high A_w.[1–4]

III. RAW AND PASTEURIZED MILK

Raw milk can come from cows, buffalo, sheep, and goats, although the largest volume comes from cows. Pasteurized or market milk includes whole, skim, low-fat, and flavored milks, as well as cream, which are heat treated (pasteurized) according to regulatory specifications.[1,2,5] Milk is high in proteins and carbohydrates (lactose), which many microorganisms can utilize for growth. Because both raw milk and pasteurized milk contain many types of bacteria as predominant microorganisms, they are refrigerated; yet they have limited shelf life.

In raw milk, microorganisms come from inside the udder, animal body surfaces, feed, air, water, and equipment used for milking and storage. The predominant types from inside a healthy udder are *Micrococcus, Streptococcus,* and *Corynebacterium*. Normally, raw milk contains $<10^3$ microorganisms/ml. If a cow has mastitis, *Streptococcus agalactiae, Sta. aureus,* coliforms, and *Pseudomonas* can be excreted in relatively high numbers. Contaminants from animals, feed, soil, and water predominantly have lactic acid bacteria; coliforms; *Micrococcus, Staphylococcus, Enterococcus, Bacillus,* and *Clostridium* spores; and Gram-negative rods. Pathogens such as *Salmonella, Lis. monocytogenes, Yer. enterocolitica*, and *Cam. jejuni* can also come from some of these sources. Equipment can be a major source of Gram-negative rods such as *Pseudomonas, Alcaligenes,* and *Flavobacterium*, as well as Gram-positive *Micrococcus* and *Enterococcus*.

During refrigerated storage (at dairy farms and processing plants) before pasteurization, only psychrotrophs can grow in raw milk. They include *Pseudomonas,*

Flavobacterium, Alcaligenes, and some coliforms and *Bacillus* spp. They can affect the acceptance quality of raw milk (e.g., by making flavor and texture undesirable). Some of them can produce heat-stable enzymes (proteinases and lipases), which can also affect the product quality, even after pasteurization of raw milk (see Chapter 20). Psychrotrophic pathogens (*Lis. monocytogenes* and *Yer. enterocolitica*) can multiply in refrigerated raw milk during storage.

Microbiological quality of raw and pasteurized milk is monitored in many countries by regulatory agencies. In the U.S., the standard plate counts of raw milk for use as market milk are $1–3 \times 10^5$/ml, and for use in product manufacturing are $0.5–1 \times 10^6$/ml. Grade A pasteurized milk can have standard plate counts of 20,000/ml and ≤10 coliforms/ml.

Microorganisms present in pasteurized milk are those that survive pasteurization of raw milk (e.g., the thermodurics) and those that enter after heating and before packaging (e.g., postpasteurization contaminants). Thermodurics surviving pasteurization include *Micrococcus*, some *Enterococcus* (e.g., *Ent. faecalis*), *Streptococcus*, some *Lactobacillus* (e.g., *Lab. viridescens*), and spores of *Bacillus* and *Clostridium*. Postheat contaminants can be coliforms as well as *Pseudomonas, Alcaligenes*, and *Flavobacterium*. Some heat-sensitive pathogens can also enter pasteurized milk following heat treatment. Psychrotrophs can grow during refrigerated storage.[1,2,5]

IV. SHELL EGG AND LIQUID EGG

Shell eggs are contaminated with microorganisms on the outer surface from fecal matter, nesting materials, feeds, air, and equipment. Each shell, depending on the contamination level, can have 10^7 bacteria. Washing helps reduce bacterial level considerably. Eggshells can harbor different types of bacteria, namely *Pseudomonas, Alcaligenes, Proteus, Citrobacter, Esc. coli, Enterobacter, Enterococcus, Micrococcus*, and *Bacillus*. They can also have *Salmonella* from fecal contamination. Infected ovaries of laying hens can be the source of *Salmonella* Enteritidis in the yolk. Liquid egg can be contaminated with bacteria from the shell of washed eggs as well as from the breaking equipment, water, and air. Pasteurization can reduce the numbers to 10^3/ml. Bacteria, especially motile Gram-negative bacteria, can enter through pores of eggshells, particularly if the shells are wet. Several antimicrobial factors present in egg albumin, such as lysozyme, conalbumin (binds iron), avidin (binds biotin), or alkaline pH (8.0 to 9.0), can control bacterial growth. However, if the storage temperature is favorable, they can grow in yolk that is rich in nutrients and has a pH of 7.0. Pasteurization of liquid egg has been designed to destroy pathogens (especially *Salmonella*) and other Gram-negative rods. Thermoduric bacteria, namely *Micrococcus, Enterococcus* and *Bacillus*, present in the raw liquid egg survive pasteurization.[1,2,6]

V. FISH AND SHELLFISH

This group includes finfish, crustaceans (shrimp, lobster, crabs), and mollusks (oysters, clams, scallops) harvested from aquatic environments (marine and freshwa-

ter).[1,2,7] Fish and shellfish are harvested from natural sources and aquacultures. In general, they are rich in protein and nonprotein nitrogenous compounds; their fat content varies with type and season. Except for mollusks, they are very low in carbohydrates; mollusks contain about 3% glycogen.

The microbial population in these products varies greatly with the pollution level and temperature of the water. Bacteria from many groups, as well as viruses, parasites, and protozoa, can be present in the raw materials. Muscles of fish and shellfish are sterile, but scales, gills, and intestines harbor microorganisms. Finfish and crustaceans can have 10^{3-8} bacterial cells/g. During feeding, mollusks filter large volumes of water and can thus concentrate bacteria and viruses. Products harvested from marine environments can have halophilic vibrios as well as *Pseudomonas, Alteromonas, Flavobacterium, Enterococcus, Micrococcus,* coliforms, and pathogens such as *Vib. parahaemolyticus, Vib. vulnificus,* and *Clo. botulinum* type E. Freshwater fish generally have *Pseudomonas, Flavobacterium, Enterococcus, Micrococcus, Bacillus*, and coliforms. Fish and shellfish harvested from water polluted with human and animal waste can contain *Salmonella, Shigella, Clo. perfringens, Vib. cholerae*, and hepatitis A and Norwalk-like viruses. They can also contain opportunistic pathogens such as *Aeromonas hydrophila* and *Plesiomonas shigelloides*. The harvesting of seafoods, especially shellfish, is controlled by regulatory agencies in the U.S. Water with high coliform populations is closed to harvest.

Following harvest, microorganisms can grow rapidly in fish and crustaceans because of high A_w and high pH of the tissue and availability of large amounts of nonprotein nitrogenous compounds. As many of the bacterial species are psychrotrophs, they can grow at refrigerated temperature. Pathogens can remain viable for a long time during storage. Microbial loads are greatly reduced during their subsequent heat processing to produce different products.

VI. VEGETABLES, FRUITS, AND NUTS

Vegetables include edible plant components such as leaves, stalks, roots, tubers, bulbs, and flowers. In general, they are relatively high in carbohydrates, with pH values of 5.0 to 7.0. Thus, different types of bacteria, yeasts, and molds can grow if other conditions are favorable.[1,2,8] Fruits are high in carbohydrates, and have a pH of 4.5 or below because of the presence of organic acids, and some also have antimicrobial essential oils. Nuts can be from the ground (peanuts) or from trees (pecans) and have protective shells and low A_w (0.7). They are converted to nutmeats for further use or to products such as peanut butter.

Microorganisms in vegetables can come from several sources, such as soil, water, air, animals, insects, birds, or equipment, and vary with the types of vegetables. A leafy vegetable has more microorganisms from the air, whereas a tuber has more from the soil. Microbial levels and types in these products also vary greatly, depending on environmental conditions and conditions of farming and harvesting. Generally, vegetables have 10^{3-5} microorganisms/cm^2 or 10^{4-7}/g. Some of the predominant bacterial types are lactic acid bacteria, *Corynebacterium, Enterobacter, Proteus, Pseudomonas, Micrococcus, Enterococcus*, and sporeformers. They also have

different types of molds, such as *Alternaria, Fusarium,* and *Aspergillus*. Vegetables can have enteric pathogens, especially if animal and human wastes and polluted water are used for fertilization and irrigation. They include *Lis. monocytogenes, Salmonella, Shigella, Campylobacter, Clo. botulinum*, and *Clo. perfringens*. They can also have pathogenic protozoa and parasites. If the vegetables are damaged, then plant pathogens (e.g., *Erwinia*) can also predominate. Many of the microorganisms can cause different types of spoilage (different types of rot) of raw products. Pathogens can grow in plant products and cause foodborne diseases (e.g., listeriosis or botulism). Lactic acid bacteria have important roles in the natural fermentation of vegetables (e.g., sauerkraut). Different methods used to process vegetables and vegetable products greatly reduce the microbial population.

Fruits, because of their high carbohydrate content and low pH, favor the growth of different types of molds, yeasts, and lactic acid bacteria. Microorganisms generally come from air, soil, insects, and harvesting equipment. In general, microbial populations are 10^{3-6}/g. Improperly harvested and processed fruits can have pathogens that survive, grow, and cause foodborne disease. Molds, yeasts, and bacteria can cause different types of spoilage. Natural flora, especially yeasts in fruits, can be important in alcohol fermentation.

Microorganisms enter nuts from soil (peanuts) and air (tree nuts). During processing, air, equipment, and water can also be the sources. Nuts are protected by shells, but damage on the shell can facilitate microbial contamination. Raw nuts and nutmeats can have 10^{3-4} microorganisms/g, with *Bacillus* and *Clostridium* spores, *Leuconostoc, Pseudomonas*, and *Micrococcus* predominating. Because of a low A_w, bacteria do not grow in the products. However, when used as ingredients, they can cause microbiological problems in the products. Molds can grow in nuts and nutmeats and produce mycotoxins (from toxin-producing strains such as aflatoxins by *Aspergillus flavus*).

VII. CEREAL, STARCHES, AND GUMS

Cereal includes grains, flour, meals, breakfast cereals, pasta, baked products, dry mixes, and frozen and refrigerated products of cereal grains (also beans and lentils). Starches include flours of cereals (e.g., corn, rice), tapioca (from plant), potatoes, and other tubers. Gums are used as stabilizers, gelling agents, and film, and are obtained from plants, seaweeds, and microorganisms (e.g., tragacanth, pectin, xanthan, agar, and carrageenan) and as modified compounds (e.g., carboxymethyl cellulose). They are rich in amylose and amylopectin, but can also have simple sugars (e.g., in grains) and protein (e.g., in lentils). Microbial sources are mainly the soil, air, insects, birds, and equipment.[1,2]

Unprocessed products (grains) may contain high bacterial levels (aerobic plate count $\cong 10^4$/g, coliform $\cong 10^2$/g, yeasts and molds $\cong 10^3$/g). They may also contain mycotoxins produced by toxicogenic molds. Processed products may also contain a wide variety of yeasts, molds, and bacteria. Flours and starches may have higher microbial counts, similar to those of grains, whereas processed products (such as breakfast cereals and pasta) may contain aerobic plate count of 10^{2-3}/g, coliform of

$<10^{1-2}$/g, and yeasts and molds of $<10^{1-2}$/g. They can contain bacterial spores and psychrotrophs. Some pathogens, such as *Salmonella, Sta. aureus*, and *Clo. perfringens*, have also been isolated. Depending on the product, they can either grow (such as in dough) or be the source (when used as ingredients) for spoilage and pathogenic microorganisms as well as mycotoxins. Gums also may be the source of yeasts, molds (also mycotoxins), bacterial spores, and lactic acid bacteria.

VIII. CANNED FOODS

This group of foods includes those packed in hermetically sealed containers and given high heat treatment. The products with a pH of 4.6 or above are given heat treatments to obtain commercial sterility, but those with a pH below 4.6 are given heat treatments ca. 100°C.[1,2]

Canned foods prepared and processed to obtain commercial sterility can have spores of thermophilic spoilage bacteria, namely *Bacillus stearothermophilus, Clo. thermosaccharolyticum,* and *Desulfotomaculum nigrificans*. Their major sources in the products are soil and blanching water as well as sugar and starches used as ingredients. In canned products stored at 30°C or below, thermophilic spores do not germinate to cause spoilage. However, if the cans are temperature-abused to 40°C or higher, the spores germinate; subsequently, the cells multiply and spoil the products.

If the canned products are given lower heat treatment (ca. 100°C), spores of mesophilic bacteria that include both spoilage (*Bac. coagulans, Bac. licheniformis, Clo. sporogenes, Clo. butyricum*) and pathogenic types (*Bac. cereus, Clo. perfringens, Clo. botulinum*), along with the spores of thermophiles, survive. In low-pH products, particularly in tomato products, *Bac. coagulans* spores can germinate and cells can multiply and cause spoilage. Other sporeformers can germinate and grow in high-pH products. *Sta. aureus* toxins, if present in raw products, are not destroyed by the heat treatment of the canned products and can thus cause food poisoning following consumption of the products.[1,2]

IX. SUGARS AND CONFECTIONERIES

Refined sugar is obtained from sugar cane and beets. Sugar can have thermophilic spores of *Bac. stearothermophilus, Bac. coagulans, Clo. thermosaccharolyticum*, and *Des. nigrificans*, as well as mesophilic bacteria (e.g., *Lactobacillus* and *Leuconostoc*), yeasts, and molds.[1,2] When sugars are used as ingredients in food products, the spores can survive and cause spoilage of products. Pathogens are not present in refined sugar unless contaminated. In liquid sugar, mesophiles can grow. Refined sugar, used in canned products or to make liquid sugar, has strict microbiological standards (for spores).

Confectioneries include a large variety of products with a sweet taste. In general, these products have low A_w (0.84 or less) and some have low pH. They may contain many types of bacteria, yeasts, and molds, but their microbiological standards are

well regulated. Although they may harbor *Lactobacillus, Leuconostoc*, spores of *Bacillus* and *Clostridium*, and yeasts and molds, only a few osmotolerant yeasts and molds can grow. However, when used as additives in other foods, confectioneries can be a source of these microbes. If ready-to-consume products are contaminated with pathogens, either from raw materials, environment, or personnel, they can cause foodborne diseases.[1,2]

X. SOFT DRINKS, FRUIT AND VEGETABLE DRINKS, JUICES, AND BOTTLED WATER

Soft drinks are nonalcoholic beverages containing water, sweeteners, acids, flavoring, coloring and emulsifying agents, and preservatives. Some may contain fruit juices and be carbonated or noncarbonated, with a pH of 2.5 to 4.0. Fruit juices (100%) have a pH of 4.0 or below. Vegetable juices (e.g., tomato) can have a pH of 4.5 or above. Bottled water is obtained from either natural springs or drilled wells and handled under conditions that prevent contamination.

Soft drinks can have different types of microorganisms, but only aciduric microorganisms, such as molds, yeasts, lactic acid bacteria, and acetic acid bacteria, can multiply. In carbonated beverages, some yeasts being microaerophilic can grow; in beverages with fruit juices, *Lactobacillus* and *Leuconostoc* species can grow.[1,2] In noncarbonated beverages, molds (*Geotrichum*) and *Acetobacter* and *Gluconobacter* spp. can also grow. Most of these come from the processing environment and equipment. In fruit juices, molds, yeasts, *Lactobacillus* spp. (*Lab. fermentum, Lab. plantarum*), *Leuconostoc* spp. (*Leu. mesenteroides*), and acetic acid bacteria can grow. Spoilage of fruit juices by acid-resistant sporeforming species from genus *Alicyclobacillus* has currently been recognized.[9] Some pathogens (e.g., acid-tolerant *Salmonella* spp. and *Esc. coli* O157:H7 strains in orange juice and apple cider) can remain viable for a long time (≥30 d) in the acid products.[1,2,10] Vegetable juices can have molds, yeasts, and lactic acid bacteria along with *Bac. coagulans, Clo. butyricum*, and *Clo. pasteurianum*.[1,2]

Bottled water should not contain more than 10 to 100 bacteria and >10 coliforms/100 ml. The indigenous flora are mainly *Flavobacterium, Alcaligenes*, and *Micrococcus.* They may also have some *Pseudomonas* as contaminants from outside. They should not have pathogens unless produced under poor sanitation.[1,2]

XI. MAYONNAISE AND SALAD DRESSINGS

Water-in-oil emulsion products formulated with oil, water, vinegar (about 0.25% acetic acid) or lemon juice, sugar, salt, starch, gum, egg, spices, and vegetable pieces, mayonnaise and salad dressings have a pH between 3.5 and 4.0. Some low-calorie and less sour products containing less acid, less oil, and more water may have a pH of 4.5 or above.

Microorganisms are introduced into the products through ingredients, equipment, and air. However, except for aciduric microorganisms, most others die, especially when stored for a long time at room temperature. Among aciduric microorganisms, molds (*Geotrichum* and *Aspergillus* spp.), yeasts (*Saccharomyces* spp.), and several species of *Lactobacillus* (*Lab. fructivorans, Lab. brevis*) and some *Bacillus* spp. (*Bac. subtilis, Bac. mesentericus*) have been isolated.[1,2,10] Normally, their numbers should not exceed 10/g. If pathogens are introduced (e.g., *Salmonella* through eggs), they are expected to be killed rapidly; however, they may survive longer in low-calorie, high-pH products kept at refrigerated temperatures.

XII. SPICES AND CONDIMENTS

Spices are plant products (seed, flower, leaf, bark, roots, or bulb) used whole or ground, singly or mixed. Condiments are spices blended with other components and have a saucelike consistency (catsup, mustard). They are used in relatively small amounts for aroma and color.[1,2,11]

Some spices, unless given antimicrobial treatments (irradiation, because ethylene oxide is not permitted anymore), may contain microorganisms as high as 10^{6-7}/g. The most important are spores of molds, *Bacillus*, and *Clostridium* spp. Also, micrococci, enterococci, yeasts, and several pathogens such as *Salmonella* spp., *Sta. aureus,* and *Bac. cereus* have been found. They can also have mycotoxins. Although used in small amounts, they can be the source of spoilage and pathogenic microorganisms in food. Some spices such as cloves, allspice, and garlic have antimicrobial properties.

XIII. CONCLUSION

Normal microbial population in a food comes from those that enter from different sources as well as from growth of the contaminants before a food is examined. It is expected that a food that is produced under proper sanitary conditions and preserved properly will have lower microbial load. Information on normal microbial load helps determine microbiological quality of a food and also to set up microbiological standards and specifications. Mere microbial presence does not reduce the quality of food, except in the case of some pathogens. It is necessary for microorganisms to grow or multiply in a food to bring definite changes in quality. This is discussed in Chapter 5.

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QUESTIONS

1. List the psychrophilic microorganisms in raw meat. Discuss the significance of psychrophilic and facultative anaerobic microorganisms in raw chilled meats.
2. Discuss the sources of postheat contamination of low-heat-processed meat products by bacteria and their significance on the quality of these products.
3. List and discuss the significance of (a) psychrotrophic bacteria in raw milk, (b) high thermoduric bacteria in raw milk to be used for pasteurized grade A milk, and (c) >10 coliforms/ml in pasteurized grade A milk.
4. Discuss the significance of *Salmonella* contamination, with emphasis on the presence of *Salmonella* Enteriditis in shell eggs.
5. "A large volume of seafoods consumed in the U.S. and other developed countries is obtained from countries where the level of sanitation is not very stringent." Explain how the situation in this statement can affect the microbiological quality and microbiological safety of these seafoods.
6. Many vegetables are eaten raw. Discuss what microbiological concerns the consumer should have for these vegetables.
7. Molds can grow on cereal grains, peanuts, and spices at different stages of their production and processing. What concerns should consumers, regulatory agencies, and food processors have for the use of these products in foods?
8. Discuss the normal microbial quality of soft drinks, fruit juices, vegetable juices, and bottled water.
9. "Low-pH products, such as mayonnaise, can cause foodborne disease." Describe under what conditions this is possible.

10. List the microorganisms that are able to survive in properly processed canned foods and discuss their significance on the product quality.
11. Some food ingredients are generally used in small quantities in the preparation of processed foods. Even then their microbiological quality is considered important for the quality of the processed product. Name two such ingredients and briefly discuss why their microbiological quality is important.
12. Briefly explain the importance of knowing the normal microbiological quality of food.

SECTION II

Microbial Growth Response in the Food Environment

Microorganisms present in raw and processed (nonsterile and commercially sterile) foods are important for their involvement in foodborne diseases, food spoilage, and food bioprocessing. These are generally accomplished through the growth of microorganisms (except for viruses and protozoa) in foods. Growth or cell multiplication of bacteria, yeasts, and molds is influenced by the intrinsic and extrinsic environments of the food. Microbial growth is also facilitated through the metabolism of some food components that provide the needed energy and cellular materials and substrates for many by-products. Microbial growth in laboratory media is also important for quantitative and qualitative detection of the microbiological quality of a food. In this section, the nature of microbial growth and influencing factors associated with microbial growth are discussed. The process and importance of spore formation by some bacterial species and alteration in characteristics of bacterial cells under stress are also discussed. An understanding of these factors is helpful in designing methods to control (as against spoilage and health hazard), stimulate (as in bioprocessing), or detect the microorganisms in food. In this section the following aspects are discussed:

Chapter 5: Microbial Growth Characteristics
Chapter 6: Factors Influencing Microbial Growth in Food
Chapter 7: Microbial Metabolism of Food Components
Chapter 8: Microbial Sporulation and Germination
Chapter 9: Microbial Stress Response in the Food Environment

CHAPTER 5

Microbial Growth Characteristics

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I. INTRODUCTION

Microorganisms grow or multiply in numbers when exposed to a favorable environment such as food. Their growth is associated with food spoilage, foodborne diseases, and food bioprocessing. Growth is also important to isolate an unknown microbial strain involved in food spoilage, foodborne diseases, or food bioprocessing, in pure form, and study its morphological, physiological, biochemical, and genetic characteristics in order to design methods to control or stimulate its growth in food, destroy it, or improve its genetic makeup for better use.

II. MICROBIAL REPRODUCTION OR GROWTH

A. Binary Fission

An increase in number or mass of vegetative cells of bacteria, yeasts, and molds is customarily used to reflect growth for microorganisms. Bacteria reproduce by a process called transverse binary fission, or, simply, binary fission. The process includes several steps, such as DNA replication and separation, partition between the DNA, invagination and septum formation of wall and cell separation. In this process, one cell asexually divides into two cells, each an essentially true replica of the original cell. However, when growing for a long time, the population from a single cell may contain a few variant cells. In bacteria, a form of sexual recombination can occur that involves transfer of genetic materials from a donor to a recipient cell (e.g., conjugation). However, this is quite different from sexual reproduction, which is facilitated through the union of two cells (gametes) of opposite mating type.[1,2]

A bacterial cell has a specific surface area-to-volume (s/v) ratio. A newly divided cell has a higher s/v ratio, which helps in the rapid transport of nutrients from the environment. A young cell predominantly uses nutrients to synthesize energy and cellular components, leading to an increase in cell size. As the cell size increases, the s/v ratio decreases, which adversely affects the transport of nutrients into and by-products out of the cell. To increase the s/v ratio, the cell initiates division by forming constriction on the cell surface, followed by formation of a transverse wall, separating the cellular materials (especially the genetic materials) equally between two cells (Figure 5.1). The division can occur in one or more planes, depending on the species and the arrangement of the cells. After division, the two cells may separate from each other. In some species, the two cells remain attached to each other and continue to divide in one plane (e.g., in *Streptococcus* and *Bacillus* spp.). If the cells divide in two or more planes and the cells remain close to each other, they form a cluster (e.g., in *Staphylococcus* spp.). In some species, such as *Pediococcus*, cell division occurs in two or more planes, and the cells have a tendency to stay together for some time, giving rise to four- (tetrad) or eight-cell arrangements.

Yeasts and molds can also reproduce asexually. A yeast cell produces a bud that initially is much smaller in size and remains attached to the surface of the original cell. As it grows in size, it also can produce a bud, giving an appearance of a chain of buds growing on the surface of the original yeast cell. A yeast cell can have several buds forming on its surface. As the buds mature, they separate from the original cell. Molds can grow in size by cell division or elongation at the tip of a hyhpa. They can also form large numbers of asexual spores on specialized structure. Both yeasts and molds can also reproduce sexually. Viruses do not reproduce by themselves. Instead, they attach on the surface and inject their nucleic acid inside specific host cells, which then replicate the viral nucleic acid and produce viral particles. The viral particles are released into the environment following lysis of the host cells. Viruses associated with foodborne diseases do not increase in numbers by replication in food. However, if the specific host bacterial cells and the bacteriophages are present in a food, replication can occur.

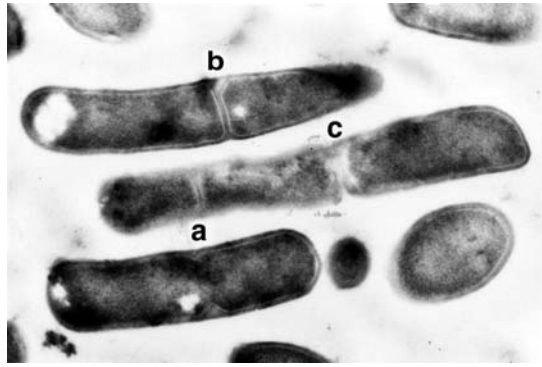


Figure 5.1 Photograph of thin sections of *Lactobacillus* cells by transmission electron microscopy showing cell wall formation during cell division at earlier stage (a), later stage (b), and final stage with partial separation of cells (c).

B. Generation Time (or Doubling Time)

The time that a single cell takes to divide into two is called generation time.[1] However, in practice, generation time is referred to as the doubling time for the entire population. In a population of a microbial species, not all cells divide at the same time or at the same rate. The generation time of a microbial species under different conditions provides valuable information for developing methods to preserve foods. In general, under optimum conditions of growth, bacteria have the shortest generation time, followed by yeasts and molds. Also, among bacterial species and strains, generation time under optimum conditions varies greatly; some species, such as *Vibrio parahaemolyticus*, under optimum conditions can have a generation time as low as 10 to 12 min. Generally, in food systems, microorganisms have longer generation times than in a nutritionally rich bacteriological broth.

The generation time of a microbial population can be calculated mathematically from the differences in population during a given time period. Because of large numbers, the calculation is done in logarithmatics ($\log_{10}$) by using the formula:

$$G = \frac{0.3t}{\log_{10} z - \log_{10} x}$$

where G is generation time (with time unit in minutes; also expressed as doubling time, t_d, with time unit usually in hours), 0.3 is a constant (value of $\log_{10}2$ and indicates doubling), t is the duration of study (min), $\log_{10}x$ is initial and $\log_{10}z$ is final cell numbers per milliliter or colony-forming units, (CFUs), per milliliter. For example, if, under a given growth condition, the initial population of 10^4 cells/ml of a bacterial species increases to 10^6 cells/ml in 120 min, its generation time will be:

$$G = \frac{0.3\text{x}120}{6 - 4} = 18\text{min}$$

For the same bacterial strain, this value changes by changing the growth conditions.

C. Specific Growth Rate

The rate of growth of microorganisms during exponential growth phase can also be determined mathematically by measuring cell numbers, $OD_{600\ nm}$, cell mass (wet or dry weight), or cell constituents (proteins, RNA, or DNA). If N_0 at time t_0 and N_t at time t are the quantity and time of any of these components, respectively, the growth rate of microorganisms is determined by the equation:

$$N_t = N_0 e^{\mu(t-t_0)}$$

where μ is specified growth rate in unit time (e.g., h^{-1}). The equation can be logarithmically expressed as:

$$\ln \frac{N_t}{N_0} = \mu(t - t_0)$$

$$\log N_t - \log N_0 = \frac{\mu}{2.3}(t - t_0)$$

$$\mu = \frac{2.3(\log N_t - \log N_0)}{t - t_0}$$

μ (h^{-1}) varies with microbial types, species, and growth environment. Normally it is ca. 0.2 for molds and yeasts. A fast-growing bacterial strain under optimum conditions can have a μ (h^{-1}) of 2.5 or higher. Under nonoptimal growth conditions, μ (h^{-1}) can range between 0.2 to 0.02. The value of doubling time (t_d) can also be determined from the μ value by the relationship $t_d/h = 0.69/\mu$ (0.69 is the value of ln2). These equations are important for determining predictable growth rate and population level (or of other components) in fermentation and shelf life of foods.

D. Optimum Growth

Many environmental parameters of food, such as storage temperature, acidity (pH), water activity (A_w), oxidation–reduction (O–R) potential, and nutrients, influence microbial growth rate. This aspect is discussed in Chapter 6. If one of the factors (e.g., temperature) is varied, keeping all other parameters constant during growth of a microbial strain, and its growth rate is measured, it is evident that the growth rate is fastest (or generation time is shortest) at a certain temperature. This temperature is referred to as the optimum growth temperature for the strain under a given

condition. The growth rate slows down on either side of the optimum growth temperature until the growth stops. The area under the two points on both sides of an optimum growth condition where minimum growth occurs is the growth temperature range. When the cells of a microbial species are exposed to a factor (e.g., temperature) beyond the growth range, the cells not only stop growing, but, depending on the situation, may be injured or lose viability. The growth range and optimum growth of a microorganism under a specific parameter provide valuable information for its inhibition, reduction, or stimulation of growth in a food.

E. Growth Curve

The growth rate and growth characteristics of a microbial population under a given condition can be graphically represented by counting cell numbers, enumerating CFUs, or measuring optical density in a spectrophotometer at a given wavelength (above 300 nm, usually at 600 nm) of a cell suspension. Cell mass, or specific cell components such as proteins, RNA, or DNA, can also be measured to determine growth rate. Each method has several advantages and disadvantages. If the CFU values are enumerated at different times of growth and a growth curve is plotted using $\log_{10}$ CFU vs. time ($\log_{10}$ CFU is used because of high cell numbers), a plot similar to the one presented in Figure 5.2 is obtained. The plot has several features that represent the conditions of the cells at different times. Initially, the population does not change (lag phase). During this time, the cells assimilate nutrients and increase in size. Although the population remains unchanged because of change in size, both cell mass and optical density show some increase. Following this, the cell number starts increasing, first slowly and then very rapidly. The cells in the population differ initially in metabolic rate and only some multiply, and then almost all cells multiply. This is the exponential phase (also called logarithmic phase). Growth rate at the exponential phase follows first-order reaction kinetics and can be used to determine generation time. Following this, the growth rate slows down and finally the population enters the stationary phase. At this stage, because of nutrient shortage and accumulation of waste products, a few cells die and a few cells multiply, keeping the living population stable. However, if one counts the cells under a microscope or measures cell mass, both may show an increase, as dead cells may remain intact. After the stationary phase, the population enters the death phase, in which the rate of cell death is higher than the rate of cell multiplication. Depending on the strain and conditions of the environment, after a long period of time (may even be a few years) some cells may still remain viable. This information is important to determine some microbiological criteria of food, especially in controlling spoilage and pathogenic microorganisms in food. It is important to note that by changing some environmental parameters (e.g., refrigeration), the growth rate of some microbial species can be slowed down, but after a long time, the population can reach high numbers to cause problems in a food. Dead cells, by the action of autolytic enzymes, may lyse and release the cellular enzymes in a food, which then can act on food components.

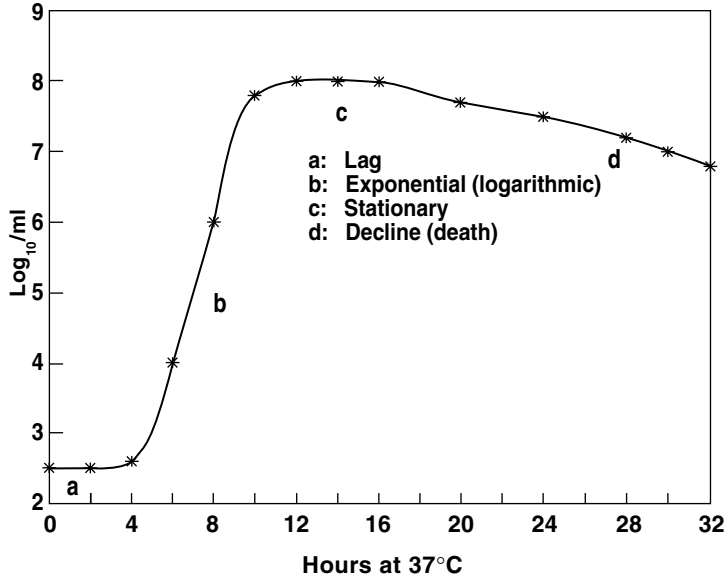


Figure 5.2 Bacterial growth curve showing changes in cell numbers of *Pediococcus acidilactici* H during 32 h of incubation at 37°C in a broth. After a 4-h lag, the cells grow exponentially up to ca. 10 h and then remain in stationary phase up to ca. 16 h before entering the death phase.

III. NATURE OF MICROBIAL GROWTH IN FOOD

A. Mixed Population

Normally, a food harbors a mixed population of microorganisms that can include different species and strains of bacteria, yeasts, and molds.[3,4] Some species can be present in relatively higher numbers than others. The growth characteristics of a mixed population differ in several respects from that of a pure culture (a single strain of a species). Depending on the environment, which includes both the food environment (intrinsic) and the environment in which the food is stored (extrinsic), some of the species or strains can be in optimum or near-optimum growth condition. Because of rapid rate of growth during storage, they will outnumber the others and become predominant. This can occur even if they are initially present in low numbers. This is often the case in foods kept for a long time under a specific condition, such as at refrigerated temperature. If the product is enumerated initially, it may show that the majority of microbial populations is able to grow at 35°C and only a few grow at 4°C (refrigerated temperature). If the product is enumerated after a few weeks of refrigerated storage, usually populations that grow at 4°C outnumber those that grow at 35°C but not at 4°C. Another situation can arise if a food contains, among the mixed population, two species initially present in equal numbers and both growing optimally under the specific intrinsic and extrinsic environments of

the food, but one having a shorter generation time than the other. After a storage period, the one with shorter generation time becomes predominant. Many foods most often get spoiled by bacteria than by yeasts and molds because, in general, bacteria have shorter generation time. Thus, in a mixed population, the intrinsic and extrinsic environments dictate which one, two, or a few in the initial mixed population will become predominant and produce specific changes in a food. These aspects are very important in the control of microbial spoilage of foods and in the production of bioprocessed (or fermented) foods.[3,4]

B. Sequence of Growth

Among the different microbial types normally present in a food, different species (strains) can become predominant in sequence during storage.[3,4] Initially, depending on the environment, one or two types may grow optimally and create an environment in which they can no longer grow rapidly. However, another type in the mixed population can find this changed environment favorable for growth and grow rather rapidly. This shift in predominance can occur several times during the storage of a food. The sequential microbial growth can be seen particularly in foods stored for a long time. If a food is packaged in a bag with a little air (e.g., ground meat), the aerobes grow first and utilize the oxygen. The environment then becomes anaerobic, in which the anaerobes (or facultative anaerobes) grow favorably. In the natural fermentation of some foods, such as sauerkraut, four different bacterial species grow in succession, one creating the favorable growth condition for the next. The desirable characteristics of the final product are dependent on the growth of all four species in specific sequence. To identify the existence of such a situation, it is necessary to enumerate and determine the microbial types at different stages of storage or fermentation.[3,4]

C. Growth in Succession or Diauxic Growth

Microorganisms that can metabolize two or more nutrients in a food, one preferred over the other and present in limiting concentrations, show growth in stages separated by a short lag phase.[1] Initially a bacterial strain grows by utilizing the preferred nutrient and after a short lag of adaptation grows by utilizing the other nutrient. During each stage, the growth curve has exponential and stationary phases with the lag phase in-between. An example is the growth of certain bacterial strains (such as some lactic acid bacteria and Gram-negative bacteria) in fresh meat. A strain grows initially by utilizing the limiting concentrations of carbohydrate present, followed by utilization of nonprotein nitrogenous (NPN; such as amino acids) substances.

D. Symbiotic Growth

Symbiosis, or helping one another, during growth often occurs in food containing two or more types of microorganisms.[3,4] One type may produce metabolic products that the second type needs for proper growth, but cannot produce by itself. In turn, the second species produces a nutrient that stimulates the first one to grow better.

This is found in the production of some fermented foods such as yogurt. Initially, *Streptococcus thermophilus* hydrolyzes milk proteins by its extracellular proteinases and generates amino acids, which are necessary for good growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Lactobacillus*, in turn, produces formate, which stimulates the growth of *Streptococcus* species. Both are necessary to produce a desirable product.[3,4]

E. Synergistic Growth

This is observed during symbiotic growth of two or more microbial types in a food.[3,4] In synergistic growth, each type is capable of growing independently and producing some metabolites at lower rates. However, when the types are allowed to grow in a mixed population, both the growth rate and the level of by-product formation greatly increase. The increase is more than the additive of the amounts produced by growing the two separately. For example, both *Str. thermophilus* and *Lab. delbrueckii* subsp. *bulgaricus*, when growing in milk independently, produce ca. 8 to 10 ppm acetaldehyde, the desirable flavor component of yogurt. However, when growing together in milk, 30 ppm or more of acetaldehyde is produced, which is much higher than the additive amounts produced independently by the two species and is necessary for a good-quality yogurt. In the production of a desirable fermented food, two separate strains and species can be used to induce synergistic growth.[3,4]

F. Antagonistic Growth

Two or more types of microorganisms present in a food can adversely affect the growth of each other, or one can interfere with the growth of one or more types; sometimes one can kill the other. This has been found among many bacterial strains, between bacteria and yeasts, between yeasts and molds, and between bacteria and molds. This occasionally occurs due to the production of one or more antimicrobial compounds by one or more strains in the mixed population. Some Gram-positive bacteria produce antibacterial proteins or bacteriocins that can kill many other types of Gram-positive bacteria. Similarly, some yeasts can produce wall-degrading enzymes and reduce the growth of molds. Some strains have probably developed these specific traits for a growth advantage in a mixed-population situation. There is now an interest to use this phenomenon to control growth and enhance viability loss of undesirable spoilage and pathogenic microorganisms in food.

IV. CONCLUSION

The study of microbial growth provides basic information that is important to understand the mechanisms of food spoilage, foodborne diseases, food bioprocessing and strain improvement, and their detection from food. Microorganisms are present in mixed cultures in food and can interact with each other during growth. Their growth is influenced by the environment of a food, which is discussed in Chapter 6.

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QUESTIONS

1. Describe the process of microbial growth with a diagram.
2. Define generation time as related to bacterial growth. If a pure culture of a bacterial population during incubation at 35°C in a nutritionally rich broth increases to 5×10^6/ml from an initial population of 2.5×10^2 in 300 min, what is the generation time of the strain?
3. Using the data in Question 2, determine the specific growth rate of the bacterial population.
4. A ground beef sample has 10^5 CFUs/g of Gram-negative psychrotrophic bacterial population. The specific growth rate of the population at 5°C is 0.2. In how many hours will the population reach 10^7CFUs/g? Determine its t_d value.
5. "In foods, microorganisms are present as a mixed population." What disadvantage does this situation impose in applying the results of pure-culture study in food systems?
6. Explain synergistic growth, antagonistic growth, and symbiotic growth of microorganisms in food. What are their advantages and disadvantages?
7. A food contains two nutrients that can be used by one bacterial strain present in it. Indicate how the bacterial strain will use the two nutrients.
8. List one advantage and one disadvantage of measuring microbial growth by (a) CFU counts, (b) OD at 600 nm, and (c) cell mass.

CHAPTER 6

Factors Influencing Microbial Growth in Food

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I. INTRODUCTION

The ability of microorganisms (except viruses) to grow or multiply in a food is determined by the food environment as well as the environment in which the food is stored, designated as the intrinsic and extrinsic environment of food, respectively. It is not possible to study the influence of any one factor on growth independently as the factors are interrelated. Instead, the influence of any one factor at different levels on growth is compared keeping other factors unchanged. The influence of these factors is discussed here.

II. INTRINSIC FACTORS OR FOOD ENVIRONMENT

Intrinsic factors of a food include nutrients, growth factors, and inhibitors (or antimicrobials), water activity, pH, and oxidation–reduction potential. The influence of each factor on growth is discussed separately. But, as indicated previously, in a food system the factors are present together and exert effects on microbial growth in combination, either favorably or adversely.

A. Nutrients and Growth

Microbial growth is accomplished through the synthesis of cellular components and energy.[1] The necessary nutrients for this process are derived from the immediate environment of a microbial cell and, if the cell is growing in a food, it supplies the nutrients. These nutrients include carbohydrates, proteins, lipids, minerals, and vitamins. Water is not considered a nutrient, but it is essential as a medium for the biochemical reactions necessary for the synthesis of cell mass and energy. All foods contain these five major nutrient groups, either naturally or added, and the amount of each nutrient varies greatly with the type of food.[2] In general, meat is rich in protein, lipids, minerals, and vitamins but poor in carbohydrates. Foods from plant sources are rich in carbohydrates but can be poor sources of proteins, minerals, and some vitamins. Some foods such as milk and many prepared foods have all five nutrient groups in sufficient amounts for microbial growth.

Microorganisms normally present in food vary greatly in nutrient requirements, with bacteria requiring the most, followed by yeasts and molds. Microorganisms also differ greatly in their ability to utilize large and complex carbohydrates (e.g., starch and cellulose), large proteins (e.g., casein in milk), and lipids. Microorganisms capable of using these molecules do so by producing specific extracellular enzymes (or exoenzymes) and hydrolyzing the complex molecules to simpler forms outside before transporting them inside the cell. Molds are the most capable of doing this. However, this provides an opportunity for a species to grow in a mixed population even when it is incapable of metabolizing the complex molecules. Microbial cells, following death and lysis, release intracellular enzymes that can also catalyze breakdown of complex food nutrients to simpler forms, which can then be utilized by other microorganisms.

1. Carbohydrates in Foods

Major carbohydrates present in different foods, either naturally or added as ingredients, can be grouped on the basis of chemical nature as follows:

- Monosaccharides
  - Hexoses: glucose, fructose, mannose, galactose
  - Pentoses: xylose, arabinose, ribose, ribulose, xylulose
- Disaccharides
  - Lactose (galactose + glucose)
  - Sucrose (fructose + glucose)
  - Maltose (glucose + glucose)
- Oligosaccharides
  - Raffinose (glucose + fructose + galactose)
  - Stachyose (glucose + fructose + galactose + galactose)
- Polysaccharides
  - Starch (glucose units)
  - Glycogen (glucose units)
  - Cellulose (glucose units)
  - Inulin (fructose units)
  - Hemicellulose (xylose, galactose, mannose units)
  - Dextrans (α-1, 6 glucose polymer)
  - Pectins
  - Gums and mucilages

Lactose is found only in milk and thus can be present in foods made from or with milk and milk products. Glycogen is present in animal tissues, especially in liver. Pentoses, most oligosaccharides, and polysaccharides are naturally present in foods of plant origin.

All microorganisms normally found in food metabolize glucose, but their ability to utilize other carbohydrates differs considerably. This is because of the inability of some microorganisms to transport the specific monosaccharides and disaccharides inside the cells and the inability to hydrolyze polysaccharides outside the cells. Molds are the most capable of using polysaccharides.

Food carbohydrates are metabolized by microorganisms principally to supply energy through several metabolic pathways. Some of the metabolic products can be used to synthesize cellular components of microorganisms (e.g., to produce amino acids by amination of some keto acids). Microorganisms also produce metabolic byproducts associated with food spoilage (CO_2 to cause gas defect) or food bioprocessing (lactic acid in fermented foods). Some are also metabolized to produce organic acids, such as lactic, acetic, propionic, and butyric acids, which have an antagonistic effect on the growth and survival of many bacteria. Some of these metabolic pathways are discussed in Chapter 7 and Chapter 11. Microorganisms can also polymerize some monosaccharides to produce complex carbohydrates such as dextrans, capsular materials, and cell wall (or outer membrane and middle membrane in Gram-negative bacteria). Some of these carbohydrates from pathogens may cause health hazards (forming complexes with proteins), some may cause food spoilage (such as slime

defect), and some can be used in food production (such as dextrans as stabilizers). Carbohydrate metabolism profiles are extensively used in the laboratory for the biochemical identification of unknown microorganisms isolated from foods.

2. Proteins in Foods

The major proteinaceous components in foods are simple proteins, conjugated proteins, peptides, and nonprotein nitrogenous (NPN) compounds (amino acids, urea, ammonia, creatinine, trimethylamine). Proteins and peptides are polymers of different amino acids without or with other organic (e.g., a carbohydrate) or inorganic (e.g., iron) components and contain ca. 15 to 18% nitrogen. Simple food proteins are polymers of amino acids, such as albumins (in egg), globulins (in milk), glutelins (gluten in cereal), prolamins (zein in grains), and albuminoids (collagen in muscle). They differ greatly in their solubility, which determines the ability of microorganisms to utilize a specific protein. Many microorganisms can hydrolyze albumin, which is soluble in water. In contrast, collagens, which are insoluble in water or weak salt and acid solutions, are hydrolyzed only by a few microorganisms. As compared with simple proteins, conjugated proteins of food on hydrolysis produce metals (metalloproteins such as hemoglobin and myoglobin), carbohydrates (glycoproteins such as mucin), phosphate (phosphoproteins such as casein), and lipids (lipoproteins such as some in liver). Proteins are present in higher quantities in foods of animal origin than in foods of plant origin. But plant foods, such as nuts and legumes, are rich in proteins. Proteins as ingredients can also be added to foods.

Microorganisms differ greatly in their ability to metabolize food proteins. Most transport amino acids and small peptides in the cells; small peptides are then hydrolyzed to amino acids inside the cells, such as in some *Lactococcus* spp. Microorganisms also produce extracellular proteinases and peptidases to hydrolyze large proteins and peptides to small peptides and amino acids before they can be transported inside the cells. Soluble proteins are more susceptible to this hydrolytic action than are the insoluble proteins. Hydrolysis of food proteins can be either undesirable (texture loss in meat) or desirable (flavor in cheese). Microorganisms can also metabolize different NPN compounds found in foods.

Amino acids inside microbial cells are metabolized via different pathways to synthesize cellular components, energy, and various by-products. Many of these by-products can be undesirable (e.g., NH_3 and H_2S production causes spoilage of food, and toxins and biological amines cause health hazards) or desirable (e.g., some sulfur compounds give cheddar cheese flavor). Production of specific metabolic products is used for the laboratory identification of microbial isolates from food. An example of this is the ability of *Escherichia coli* to produce indole from tryptophan, which is used to differentiate this species from non-indole-producing related species (e.g., *Enterobacter* spp.).

3. Lipids in Foods

Lipids in foods include compounds that can be extracted by organic solvents, some of which are free fatty acids, glycerides, phospholipids, waxes, and sterols. Lipids are

relatively higher in foods of animal origin than in foods of plant origin, although nuts, oil seeds, coconuts, and olives have high amounts of lipids. Fabricated or prepared foods can also vary greatly in lipid content. Cholesterols are present in foods of animal origin or foods containing ingredients from animal sources. Lipids are, in general, less preferred substrates for the microbial synthesis of energy and cellular materials. Many microorganisms can produce extracellular lipases, which can hydrolyze glycerides to fatty acids and glycerol. Fatty acids can be transported in cells and used for energy synthesis, whereas glycerol can be metabolized separately. Some microorganisms also produce extracellular lipid oxidases, which can oxidize unsaturated fatty acids to produce different aldehydes and ketones. In general, molds are more capable of producing these enzymes. However, certain bacterial groups such as *Pseudomonas, Achromobacter,* and *Alcaligenes* can produce these enzymes. Lysis of dead microbial cells in foods causes release of intracellular lipases and oxidases, which then can carry out these reactions. In many foods the action of these enzymes is associated with spoilage (such as rancidity), whereas in other foods the enzymes are credited for desirable flavors (such as in mold-ripened cheeses). Some beneficial intestinal microorganisms, such as *Lactobacillus acidophilus* strains, can metabolize cholesterol and are believed to be capable of reducing serum cholesterol levels in humans.

4. Minerals and Vitamins in Foods

Microorganisms need several elements in small amounts, such as phosphorous, calcium, magnesium, iron, sulfur, manganese, and potassium. Most foods have these elements in sufficient amounts. Many microorganisms can synthesize B vitamins, and foods also contain most B vitamins.

In general, most foods contain different carbohydrates, proteins, lipids, minerals, and vitamins in sufficient amounts to supply necessary nutrients for the growth of molds, yeasts, and bacteria, especially Gram-negative bacteria normally present in foods. Some foods may have limited amounts of one or a few nutrients for rapid growth of some Gram-positive bacteria, especially some fastidious *Lactobacillus* species. When their growth is desired, some carbohydrates, essential amino acids, and B vitamins may be added to a food. It is not possible or practical to control microbial growth in a food by restricting nutrients.

B. Growth Factors and Inhibitors in Food

Foods can also have some factors that either stimulate growth or adversely affect growth of microorganisms.[3] The exact nature of growth factors is not known, but they are naturally present in some foods. An example is the growth factors in tomatoes that stimulate growth of some *Lactobacillus* species. These factors can be added to raw materials during food bioprocessing or to media to isolate some fastidious bacteria from foods.

Foods also contain many chemicals, either naturally or added, that adversely affect microbial growth. Some of the natural inhibitors are lysozyme in egg, agglutinin in milk, and eugenol in cloves. The inhibitors, depending on their mode of action, can prevent or reduce growth of and kill microorganisms. This aspect is discussed in Chapter 37.

C. Water Activity and Growth

1. Principle

Water activity (A_w) is a measure of the availability of water for biological functions and relates to water present in a food in free form. In a food system, total water or moisture is present in free and bound forms. Bound water is the fraction used to hydrate hydrophilic molecules and to dissolve solutes, and is not available for biological functions; thus, it does not contribute to A_w. The A_w of a food can be expressed by the ratio of water vapor pressure of the food (P, which is <1) to that of pure water (P_o, which is 1), i.e., P_o/P. It ranges between 0 and 1, or more accurately >0 to <1, because no food can have a water activity of either 0 or 1. The A_w of a food can be determined from its equilibrium relative humidity (ERH) by dividing ERH by 100 (because ERH is expressed in percentage).[4–7]

2. A_w of Food

The A_w of food ranges from ca. 0.1 to 0.99. The A_w values of some food groups are as follows: cereals, crackers, sugar, salt, dry milk, 0.10 to 0.20; noodles, honey, chocolate, dried egg, <0.60; jam, jelly, dried fruits, parmesan cheese, nuts, 0.60 to 0.85; fermented sausage, dry cured meat, sweetened condensed milk, maple syrup, 0.85 to 0.93; evaporated milk, tomato paste, bread, fruit juices, salted fish, sausage, processed cheese, 0.93 to 0.98; and fresh meat, fish, fruits, vegetables, milk, eggs, 0.98 to 0.99. The A_w of foods can be reduced by removing water (desorption) and increased by the adsorption of water, and these two parameters can be used to draw a sorption isotherm graph for a food (Figure 6.1). The desorption process gives relatively lower A_w values than the adsorption process does at the same moisture content of a food. This has important implications in the control of a microorganism by reducing the A_w of a food. The A_w of a food can be reduced by several means, such as adding solutes, ions, hydrophilic colloids, and freezing and drying.[4–7]

3. A_w and Microbial Growth

The free water in a food is necessary for microbial growth. It is necessary to transport nutrients and remove waste materials, carry out enzymatic reactions, synthesize cellular materials, and take part in other biochemical reactions, such as hydrolysis of a polymer to monomers (proteins to amino acids). Each microbial species (or group) has an optimum, maximum, and minimum A_w level for growth. In general, the minimum A_w values for growth of microbial groups are as follows: most molds, 0.8, with xerophilic molds as low as 0.6; most yeasts, 0.85, with osmophilic yeasts, 0.6 to 0.7; most Gram-positive bacteria, 0.90; and Gram-negative bacteria, 0.93. Some exceptions are growth of *Staphlococcus aureus* at 0.85 and halophilic bacteria at 0.75. The A_w need for spore-forming bacteria to sporulate and the spores to germinate and the toxin-producing microorganisms to produce toxins is generally higher than the minimum A_w for their growth. Also, the minimum A_w for growth in an ideal condition is lower than that in a nonideal condition. As an example, if

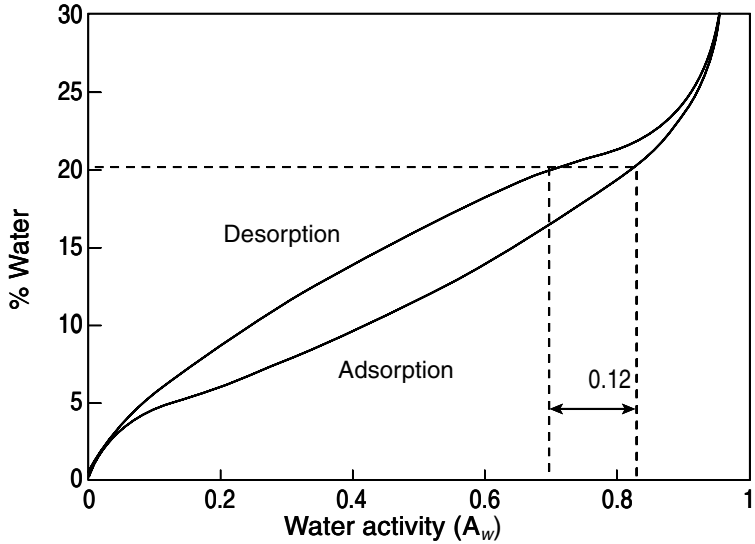


Figure 6.1 Water sorption isotherm showing hysterisis. At the same percentage of water, A_w is lower by desorption than by adsorption.

minimum A_w for growth of a bacterial strain at pH 6.8 is 0.91, then at pH 5.5, it can be 0.95 or more. When the A_w is reduced below the minimum level required for growth of a microorganism, the cells remain viable for a while. But if the A_w is reduced drastically, microbial cells in a population lose viability, generally rapidly at first and then more slowly. This information is used to control spoilage and pathogenic microorganisms in food as well as enhance the growth of desirable types in food bioprocessing (such as adding salt in processing of cured ham; see Chapter 34) and in laboratory detection of microorganisms (adding salt to media to enumerate *Sta. aureus*).

D. pH and Growth

1. Principle

pH indicates the hydrogen ion concentrations in a system and is expressed as –log $[H^+]$, the negative logarithm of the hydrogen ion or proton concentration. It ranges from 0 to 14, with 7.0 being neutral pH. $[H^+]$ concentrations can differ in a system, depending on what acid is present. Some strong acids used in foods, such as HCl and phosphoric acid, dissociate completely. Weak acids, such as acetic or lactic acids, remain in equilibrium with the dissociated and undissociated forms:

$$[HCl] \rightarrow [H^+] + [Cl^-], \text{ pH of 0.1 } N \text{ HCl is 1.1}$$

$$CH_3\ COOH\ [H^+] + [CH_3COO^-],\ \text{pH of 0.1 } N\ CH_3COOH \text{ is 2.9}$$

Acidity is inversely related to pH: a system with high acidity has a low pH, and vice versa.[8,9]

2. pH of Food

Depending on the type, the pH of a food can vary greatly. On the basis of pH, foods can be grouped as high-acid foods (pH below 4.6) and low-acid foods (pH 4.6 and above). Most fruits, fruit juices, fermented foods (from fruits, vegetables, meat, and milk), and salad dressings are high-acid (low-pH) foods, whereas most vegetables, meat, fish, milk, and soups are low-acid (high-pH) foods. Tomato, however, is a high-acid vegetable (pH 4.1 to 4.4). The higher pH limit of most low-acid foods remains below 7.0; only in a few foods, such as clams (pH 7.1) and egg albumen (pH 8.5), does the pH exceed 7.0. Similarly, the low pH limit of most high-acid foods remains above 3.0, except in some citrus fruits (lemon, lime, grapefruit) and cranberry juice, in which the pH can be as low as 2.2. The acid in the foods can either be present naturally (as in fruits), produced during fermentation (as in fermented foods), or added during processing (as in salad dressings). Foods can also have compounds that have a buffering capacity. A food such as milk or meat, because of good buffering capacity, does not show pH reduction when compared with a vegetable product in the presence of the same amount of acid.

3. pH and Microbial Growth

The pH of a food has a profound effect on the growth and viability of microbial cells. Each species has an optimum and a range of pH for growth. In general, molds and yeasts are able to grow at lower pH than do bacteria, and Gram-negative bacteria are more sensitive to low pH than are Gram-positive bacteria. The pH range of growth for molds is 1.5 to 9.0; for yeasts, 2.0 to 8.5; for Gram-positive bacteria, 4.0 to 8.5; and for Gram-negative bacteria, 4.5 to 9.0. Individual species differ greatly in lower pH limit for growth; for example, *Pediococcus acidilactici* can grow at pH 3.8 and *Sta. aureus* can grow at pH 4.5, but normally *Salmonella* cannot. The lower pH limit of growth of a species can be a little higher if the pH is adjusted with strong acid instead of a weak acid (due to its undissociated molecules). Acid-resistant or tolerant strains can acquire resistance to lower pH compared with the other strains of a species (e.g., acid-resistant *Salmonella*).

When the pH in a food is reduced below the lower limit for growth of a microbial species, the cells not only stop growing but also lose viability, the rate of which depends on the extent of pH reduction. This is more apparent with weak acids, especially with those that have higher dissociation constant (pK), such as acetic acid vs. lactic acid (with pK values 4.8 and 3.8, respectively). This is because at the same pH, acetic acid has more undissociated molecules than lactic acid does. The undissociated molecules, being lipophilic, enter into the cell and dissociate to generate H^+ in the cytoplasm. This causes a reduction in internal pH, which ultimately destroys the proton gradient between the inside and the outside of the cells and dissipates

proton motive force as well as the ability of the cells to generate energy. The information on the influence of pH on growth and viability of microbial cells is important to develop methods to prevent the growth of undesirable microorganisms in food (e.g., in acidified foods; see Chapter 35), used to produce some fermented foods (e.g., sequential growth of lactic acid bacteria in sauerkraut fermentation), and to selectively isolate aciduric microorganisms from food (e.g., yeasts and molds in a medium with pH 3.5).[8,9] Acquired acid tolerance by pathogens and spoilage bacteria can impose problems in their control in low-pH foods; this aspect is discussed in Chapter 9.

E. Redox Potential, Oxygen, and Growth

1. Principle

The redox or oxidation–reduction (O–R) potential measures the potential difference in a system generated by a coupled reaction in which one substance is oxidized and a second substance is reduced simultaneously. The process involves the loss of electrons from a reduced substance (thus it is oxidized) and the gain of electrons by an oxidized substance (thus it is reduced). The electron donor, because it reduces an oxidized substance, is also called a reducing agent. Similarly, the electron recipient is called an oxidizing agent. The redox potential, designated as Eh, is measured in electrical units of millivolts (mV). In the oxidized range, it is expressed in +mV, and in reduced range in –mV. In biological systems, the oxidation and reduction of substances are the primary means of generating energy. If free oxygen is present in the system, then it can act as an electron acceptor. In the absence of free oxygen, oxygen bound to some other compound, such as NO_3 and SO_4, can accept the electron. In a system where no oxygen is present, other compounds can accept the electrons. Thus, presence of oxygen is not a requirement of O–R reactions.[10]

2. Redox Potential in Food

The redox potential of a food is influenced by its chemical composition, specific processing treatment given, and its storage condition (in relation to air). Fresh foods of plant and animal origin are in a reduced state, because of the presence of reducing substances such as ascorbic acid, reducing sugars, and –SH group of proteins. Following stoppage of respiration of the cells in a food, oxygen diffuses inside and changes the redox potential. Processing, such as heating, can increase or decrease reducing compounds and alter the Eh. A food stored in air will have a higher Eh (+mV) than when it is stored under vacuum or in modified gas (such as CO_2 or N_2). Oxygen can be present in a food in the gaseous state (on the surface, trapped inside) or in dissolved form.

3. Redox Potential and Microbial Growth

On the basis of their growth in the presence and absence of free oxygen, microorganisms have been grouped as aerobes, anaerobes, facultative anaerobes, or

microaerophiles. Aerobes need free oxygen for energy generation, as the free oxygen acts as the final electron acceptor through aerobic respiration (see Chapter 7). Facultative anaerobes can generate energy if free oxygen is available, or they can use bound oxygen in compounds such as NO_3 or SO_4 as final electron acceptors through anaerobic respiration. If oxygen is not available, then other compounds are used to accept the electron (or hydrogen) through (anaerobic) fermentation. An example of this is the acceptance of hydrogen from $NADH_2$ by pyruvate to produce lactate. Anaerobic and facultative anaerobic microorganisms can only transfer electrons through fermentation. Many anaerobes (obligate or strict anaerobes) cannot grow in the presence of even small amounts of free oxygen as they lack the superoxide dismutase necessary to scavenge the toxic oxygen free radicals. Addition of scavengers, such as thiols (e.g., thiolglycolate), helps overcome the sensitivity to these free radicals. Microaerophiles grow better in the presence of less oxygen.

Growth of microorganisms and their ability to generate energy by the specific metabolic reactions depend on the redox potential of foods. The Eh range at which different groups of microorganisms can grow are as follows: aerobes, +500 to +300 mV; facultative anaerobes, +300 to +100 mV; and anaerobes, +100 to –250 mV or lower. However, this varies greatly with concentrations of reducing components in a food and the presence of oxygen. Molds, yeasts, and *Bacillus, Pseudomonas, Moraxella*, and *Micrococcus* genera are some examples that have aerobic species. Some examples of facultative anaerobes are the lactic acid bacteria and those in the family *Enterobacteriaceae*. The most important anaerobe in food is *Clostridium*. An example of a microaerophile is *Campylobacter* spp. The Eh range indicates that in each group some species are stricter in their Eh need than others. Although most molds are strict aerobes, a few can tolerate less aerobic conditions. Similarly, yeasts are basically aerobic, but some can grow under low Eh (below +300 mV). Many clostridial species can grow at Eh +100 mV, but some need –150 mV or less.

The presence or absence of oxygen and the Eh of food determine the growth capability of a particular microbial group in a food and the specific metabolic pathways used during growth to generate energy and metabolic by-products. This is important in microbial spoilage of a food (such as putrification of meat by *Clostridium* spp. under anaerobic conditions) and to produce desirable characteristics of fermented foods (such as growth of *Penicillium* species in blue cheese under aerobic conditions). This information is also important to isolate microorganisms of interest from foods (such as *Clostridium laramie*, a strict anaerobe from spoiled meat) in the laboratory.

III. EXTRINSIC FACTORS

Extrinsic factors important in microbial growth in a food include the environmental conditions in which it is stored. These are temperature, relative humidity, and gaseous environment. The relative humidity and gaseous condition of storage, respectively, influence the A_w and Eh of the food. The influence of these two factors on microbial growth has been discussed previously. In this section, the influence of storage temperature of food on microbial growth is discussed.

A. Temperature and Growth

1. Principle

Microbial growth is accomplished through enzymatic reactions. It is well known that within a certain range, with every 10°C rise in temperature, the catalytic rate of an enzyme doubles. Similarly, the enzymatic reaction rate is reduced to half by decreasing the temperature by 10°C. This relationship changes beyond the growth range. Because temperature influences enzyme reactions, it has an important role in microbial growth in food.

2. Food and Temperature

Foods are exposed to different temperatures from the time of production until consumption. Depending on processing conditions, a food can be exposed to high heat, from 65°C (roasting of meat) to more than 100°C (in ultrahigh temperature processing). For long-term storage, a food can be kept at 5°C (refrigeration) to –20°C or below (freezing). Some relatively stable foods are also kept between 10 and 35°C (cold to ambient temperature). Some ready-to-eat foods are kept at warm temperature (50° to 60°C) for several hours (e.g., in the supermarket deli). Different temperatures are also used to stimulate desirable microbial growth in food fermentation.[11]

3. Microbial Growth and Viability

Microorganisms important in foods are divided into three groups on the basis of their temperature of growth, each group having an optimum temperature and a temperature range of growth: (1) thermophiles (grow at relatively high temperature), with optimum ca. 55°C and range 45 to 70°C; (2) mesophiles (grow at ambient temperature), with optimum at 35°C and range 10 to 45°C; and (3) psychrophiles (grow at cold temperature), with optimum at 15°C and range –5 to 20°C. However, these divisions are not clear-cut and overlap each other.

Two other terms used in food microbiology are very important with respect to microbial growth at refrigerated temperature and survival of microorganisms to low heat treatment or pasteurization, because both methods are widely used in the storage and processing of foods. Psychrotrophs are microorganisms that grow at refrigerated temperature (0 to 5°C), irrespective of their optimum range of growth temperature. They usually grow rapidly between 10 and 30°C. Molds; yeasts; many Gram-negative bacteria from genera *Pseudomonas, Achromobacter, Yersinia, Serratia*, and *Aeromonas*; and Gram-positive bacteria from genera *Leuconostoc, Lactobacillus, Bacillus, Clostridium*, and *Listeria* are included in this group (see Chapter 20). Microorganisms that survive pasteurization temperature are designated as thermodurics. They include species from genera *Micrococcus, Bacillus, Clostridium, Lactobacillus, Pediococcus*, and *Enterococcus*. Bacterial spores are also included in this group. They have different growth temperatures and many can grow at refrigerated temperature as well as thermophilic temperature.

When the foods are exposed to temperatures beyond the maximum and minimum temperatures of growth, microbial cells die rapidly at higher temperatures and relatively slowly at lower temperatures. Microbial growth and viability are important considerations in reducing food spoilage and enhancing safety against pathogens, as well as in food bioprocessing. Temperature of growth is also effectively used in the laboratory to enumerate and isolate microorganisms from foods.

IV. CONCLUSION

The physical and chemical environments control microbial growth within the growth range mainly by influencing their metabolic process associated with synthesis of energy and cellular components. Beyond the growth range, these factors, either individually or in combination, can be used to control microbial growth and even to destroy them. Actual growth is accomplished through the metabolism of various nutrients present in a food. The processes by which the food nutrients are transported inside the microbial cells and then metabolized to produce energy, cellular molecules, and by-products are briefly discussed in Chapter 7.

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QUESTIONS

1. List the intrinsic and extrinsic factors necessary for growth of microorganisms in a food.
2. What are the major nutrients in food metabolized by the microorganisms? List the major groups of carbohydrates present in foods. List the carbohydrates in milk and meat; pentoses in plant foods; and an oligosaccharide in plant food.
3. Discuss how bacteria are able to metabolize large molecules of carbohydrates, proteins, and lipids. How do molds differ from bacteria in the metabolism of these molecules?
4. Discuss the importance of antimicrobials in foods that can adversely affect microbial growth.
5. Define A_w and explain the desorption and adsorption processes of moisture in a food. Discuss the importance of A_w in microbial growth. How do halophilic, osmophilic, and xerophilic microorganisms differ in minimum A_w need for growth?
6. Define pH and discuss factors that influence the pH of a food. Discuss the role of pH on microbial growth. How does a bacterial cell maintain a high intracellular pH (6.0) while growing in a low-pH (5.0) environment? Give examples of an aciduric bacteria and an acid-tolerant bacteria.
7. Define redox potential and discuss how it influences microbial growth in a food. How can microorganisms be grouped on the basis of their growth capabilities at different redox potentials and oxygen availabilities?
8. How are microorganisms grouped on the basis of their temperature of growth and survival? Discuss the significance of psychrotrophic and thermoduric microorganisms in the processing and refrigerated storage of foods.

CHAPTER 7

Microbial Metabolism of Food Components

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I. INTRODUCTION

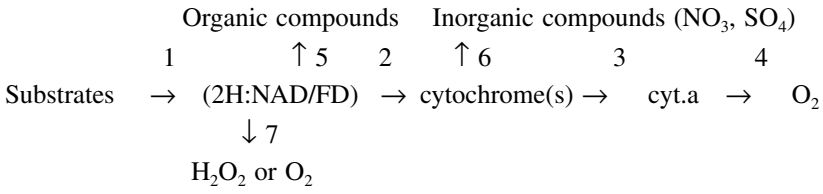
Bacterial growth in food occurs though the metabolism of food components or nutrients mainly in the cytoplasm and cytoplasmic membrane (also in periplasmic space in Gram-negative bacteria) of cells.[1] The complete process involves transport of nutrients from the environment (for macromolecules after their enzymatic breakdown) inside the cell through the cell wall and cell membrane, breakdown of nutrients to generate energy and active building blocks, synthesis of cell components from the building blocks (mainly macromolecules and structural and functional components), and release of unusable end products in the environment. In addition, recycle of cellular materials, cell differentiation, repair of injured structures, and adaptation to environmental stresses are also mediated through metabolic processes. Some of these aspects are important in food microbiology and have been briefly discussed in several sections in this book: sporulation in Chapter 8, sublethal injury and repair and stress adaptation in Chapter 9, and nutrient transport in Chapter 11. Several energy-generation and energy-degradation processes that are important in food microbiology are briefly discussed here (also in Chapter 11).

II. RESPIRATION AND FERMENTATION DURING GROWTH

During growth in a food, microorganisms synthesize energy and cellular materials. A large portion of the energy is used to synthesize cellular components. This is achieved through linking the energy-producing reactions with the reactions involved in the synthesis of cell materials. The energy-generating reactions are of the nature of oxidation, and in microorganisms they are organized in sequences (metabolic pathways) for gradual liberation of energy from an organic substrate (in reduced state). The energy is then either used directly in an endergonic reaction or stored for release during a later reaction. The energy can be stored by forming energy-rich intermediates capable of conserving the free energy as biochemical energy. Some of these are derivatives of phosphoric acid (nucleotide triphosphates, acylphosphates, and inorganic polyphosphates) and derivatives of carboxylic acids (acetyl-coenzyme A). The most important of these is ATP, which is formed from ADP in coupled reactions, through either oxidative phosphorylation or substrate-level phosphorylation.

The energy-liberating oxidation reactions of the substrates generate electrons ($H_2 \rightarrow 2H^+ + 2e^-$), which are then accepted by the oxidizing agents. This aspect is briefly discussed under redox potential in Chapter 6. In a food system, the substrates are mainly the metabolizable carbohydrates, proteins, and lipids. Microorganisms important in foods are heterotrophs (i.e., require organic carbon sources, substances more reduced than CO_2) and chemoorganotrophs (i.e., use organic compounds as electron donors to generate energy). On the basis of the nature of the terminal electron acceptors, there are different energy-generating reactions: aerobic respiration, which requires molecular oxygen as the electron acceptor; anaerobic respiration, in which inorganic compounds act as the electron acceptors; and fermentation, which uses organic compounds as electron acceptors.[1,2] The methods

used for electron transfer by different groups of microorganisms important in food can be illustrated by the following scheme:



Aerobes (some *Bacillus* spp., *Pseudomonas*, molds, and yeasts): 1,2,3,4¥
Anaerobes
  Cyt. independent, e.g., some *Clostridium* spp.: 1, 5
  Cyt. dependent, e.g., *Desulfotomaculum* spp.: 1, 2, 6
Facultative anaerobes
  Cyt. independent, e.g., lactic acid bacteria, some yeasts
    Aerobic: 1, 7
    Anaerobic: 1, 5
  Cyt. dependent, e.g., *Enterobacteriaceae* group
    Aerobic: 1, 2, 3, 4, or 1, 7
    Anaerobic: 1, 5 or 1, 2, 6

The energy-generating metabolic pathways also produce (from the substrates) many metabolic products that the microbial cells either use for the synthesis of cellular components or release into the environment. The nature of these metabolites differs greatly and depends on the nature of the substrates, the type of microorganisms with respect to their aerobiosis nature, and the oxygen availability (more correctly, redox potential) of the environment. The metabolisms and growth of microorganisms in food are important for several reasons. Microbial spoilage of foods with the loss of acceptance qualities (e.g., flavor, texture, color, and appearance) is directly related to microbial growth and metabolism. Toxin production in food by food-poisoning microorganisms also results from their growth in a food. Many microbial metabolites are also important for their ability to produce desirable characteristics in fermented foods, such as texture, flavor, and long shelf life. Microbial metabolic products are also used in foods for processing (enzymes), preservation (bacteriocins and acids), and improving texture (dextran) and flavor (diacetyl).

Among the food components, microbial metabolisms of carbohydrates, proteins, and lipids are of major importance. Some of these metabolic pathways are briefly presented in this chapter. Foods, depending on the type, can contain many types of carbohydrates, proteins, and lipids. This has been discussed briefly previously. Depending on the type and source, foods also differ greatly in the amounts of the three groups of nutrients. Plant foods are, in general, rich in carbohydrates, although some (e.g., nuts, lentils, and beans) are also rich in protein and some others (e.g., oilseeds) are rich in lipids. Foods of animal origin are rich in proteins and lipids, whereas some (e.g., meat and fish) are low in carbohydrates; others, such as milk, organ meats (liver), and mollusks (oysters), are rich in proteins as well as carbohydrates. Fabricated or formulated foods can have all the nutrients in sufficient

quantities to support microbial growth. In general, microorganisms preferentially metabolize carbohydrates as an energy source over proteins and lipids. Thus, microorganisms growing in a food rich in metabolizable carbohydrates utilize carbohydrates, but in a food low in metabolizable carbohydrates and rich in metabolizable proteins they metabolize proteins (after metabolizing the carbohydrates). In a food rich in both carbohydrates and proteins, microorganisms usually utilize the carbohydrates first, produce acids, and reduce the pH. Subsequent microbial degradation of proteins can be prevented at low pH, causing nondegradation of proteins or a protein sparing effect. In the formulation of processed meat products, added carbohydrates can provide this benefit.

III. METABOLISM OF FOOD CARBOHYDRATES

Food carbohydrates comprise a large group of chemical compounds that include monosaccharides (tetroses, pentoses, and hexoses), disaccharides, oligosaccharides, and polysaccharides. Although carbohydrates are the most preferred source of energy production, microorganisms differ greatly in their ability to degrade individual carbohydrates.[1–5] Carbohydrates that are degraded at the cellular level as monosaccharides; disaccharides, and trisaccharides can be transported inside the cell and hydrolyzed to monosaccharide units before further degradation. Polysaccharides are broken down to mono- and disaccharides by extracellular microbial enzymes (e.g., α-amylase) secreted in the environment before they can be transported and metabolized.

A. Degradation of Polysaccharides

Molds, some *Bacillus* spp. and *Clostridium* spp., and several other bacterial species can degrade starch, glycogen, cellulose, pectin, and other polysaccharides by extracellular enzymes. The mono- and disaccharides are then transported in the cell and metabolized. Breakdown of these polysaccharides, especially pectins and cellulose, in fruits and vegetables by microorganisms can affect the texture and reduce the acceptance quality of the products.

B. Degradation of Disaccharides

Disaccharides of foods, either present in food (lactose, sucrose) or produced through microbial degradation (maltose from starch), are hydrolyzed to monosaccharides inside the cell by specific enzymes: lactose by lactase to galactose and glucose, sucrose by sucrase to glucose and fructose, and maltose by maltase to glucose. Many microbial species cannot metabolize one or more disaccharides.[1–5]

C. Degradation of Monosaccharides

Monosaccharides are degraded (catabolized) by aerobic, anaerobic, and facultative anaerobic microorganisms via several pathways that generate many types of intermediate and end products. The metabolic pathways are dependent on the type and

amount of monosaccharides, type of microorganisms, and redox potential of the system. Although all microorganisms important in foods can metabolize glucose, they differ greatly in their ability to utilize fructose, galactose, tetroses, and pentoses. Fermentable monosaccharides are metabolized by five major pathways, and many microbial species have more than one pathway. They are the Embden–Meyerhoff–Parnas (EMP) pathway, the hexose monophosphate shunt (HMS) or pathway, the Entner–Doudroff (ED) pathway, and two phosphoketolase (PK) pathways (pentose phosphoketolase and hexose phosphoketolase). Pyruvic acid produced via these pathways is subsequently metabolized by microorganisms in several different pathways through fermentation, anaerobic respiration, and aerobic respiration.[1–5]

D. Fermentation

Monosaccharides are fermented by anaerobic and facultative microorganisms by the five major pathways mentioned previously.[1–3] In addition, several other pathways are used, especially for the metabolism of pyruvate, by some specific microbial species and groups. In general, the terminal electron acceptors are organic compounds, and energy is produced at the substrate level. The overall reactions of the five main pathways and the end products are briefly listed in Table 7.1 (also in Table 8.1).[1–3] The metabolic pathways are discussed in more detail in Chapter 11.

1. EMP Pathway

The EMP pathway is used by homofermentative lactic acid bacteria, *Enterococcus faecalis, Bacillus* spp., and yeasts.

$$\text{Glucose phosphate} \rightarrow \text{Fructose diphosphate} \rightarrow 2\ \text{Pyruvate}$$

$$\text{Lactate fermentation: Pyruvate} \rightarrow \text{Lactate}$$

$$\text{Alcohol fermentation: Pyruvate} \rightarrow \text{Acetaldehyde} \rightarrow \text{Ethanol}$$

$$\downarrow$$

$$CO_2$$

Table 7.1 End Products of Carbohydrate Metabolism by Some Microorganisms

Microbial Type	Fermentation Pattern	Major End Products
Yeasts	Alcohol	Ethanol, CO_2
Lactic acid bacteria	Homofermentative	Lactate
	Heterofermentative	Lactate, acetate, ethanol, CO_2, diacetyl, acetoin
Bifidobacteria	Bifidus (hexose ketolate)	Lactate, acetate
Propionibacteria	Propionic acid	Propionate, acetate, CO_2
Enterobacteriaceae	Mixed acid	Lactate, acetate, formate, CO_2, H_2, succinate
Bacillus, Pseudomonas	Butanediol	Lactate, acetate, formate, 2,3-butanediol, CO_2, H_2
Clostridium	Butyric acid	Butyrate, acetate, H_2, CO_2, butanol, ethanol, acetone, isopropanol

Other hexose monophosphates enter the EMP pathway at different steps, mostly before fructose diphosphate.

2. HMP Pathway

The HMP pathway is also called the HMP shunt, pentose cycle, or Warburg–Dickens–Horecker pathway. It is used by heterofermentative lactic acid bacteria, *Bacillus* spp., and *Pseudomonas* spp.

Glucose-phosphate → Phosphogluconate → Ribulose phosphate
↓
CO_2
→ Ribose phosphate → Acetyl phosphate + Pyruvate
Acetyl-P → Acetate or Ethanol
Pyruvate → Lactate

Ribose phosphate can be used for the synthesis of ribose and deoxyribose moieties in nucleic acids.

3. ED Pathway

The ED pathway is used by *Pseudomonas* spp.

Glucose phosphate → Phosphogluconate → Pyruvate + Glyceraldehyde phosphate
Pyruvate → Acetaldehyde → Ethanol
↓
CO_2
Glyceraldehyde phosphate → Pyruvate → Acetaldehyde → Ethanol
↓
CO_2

4. Pentose Phosphoketolase Pathway

This pathway is used by *Escherichia coli, Enterobacter aerogenes, Bacillus* spp., and some lactic acid bacteria.

Ribose phosphate → Xylulose phosphate
→ Acetyl-Phosphate + Pyruvate
→ Acetate or Ethanol + Lactate

5. Hexose Phosphoketolase Pathway

This pathway is also called Bifidus pathway and is used by *Bifidobacterium* spp.

2 Glucose phosphate → Acetyl phosphate + 2 Xylulose phosphate
→ 3 Acetate + 2 Pyruvate
→ 3 Acetate + 2 Lactate

6. Some Specific Pathways

a. Mixed Acid Fermentation

Mixed acid fermentation is characteristic of members of *Enterobacteriaceae*. Pyruvate produced from monosaccharide fermentation can be used to produce different end products.

Pyruvate → Lactate
Pyruvate → Formate + Acetyl ~ CoA
Formate → H_2 + CO_2
Acetyl ~ CoA → Acetate and Ethanol
Pyruvate → Succinate

b. Propionic Acid Fermentation

This type of fermentation is carried out by propionic bacteria and *Clostridium* spp.

Lactate → Pyruvate → Propionyl ~ CoA → Propionate (propionibacteria)
Lactate → Acrylyl ~ CoA → Propionyl ~ CoA → Propionate (*Clostridium* spp).

c. Butyrate, Butanol, and Acetone Fermentation

This type of fermentation is carried out by *Clostridium* spp.

Pyruvate → H_2 + CO_2 + Acetyl ~ CoA
Acetyl ~ CoA → Acetate, Butyrate, Butanol, and Acetone
Acetyl ~ CoA is used to produce different compounds

d. Diacetyl, Acetoin, and Butanediol Fermentation

This type of fermentation is carried out by some lactic acid bacteria and some members of *Enterobacteriaceae.*

2 Pyruvate → [Pyruvate + Acetaldehyde ~ TPP] → α Acetolactate
↓
CO_2
$2H^+$ $2H^+$
CO_2
↑ ↓ ↓
→ Diacetyl → Acetoin → 2,3-Butanediol

E. Anaerobic Respiration

Sulfate-reducing *Desulfatomaculum nigrificans* metabolizes glucose as an energy source, primarily through the EMP pathway, to produce pyruvate, which is then decarboxylated to generate acetate (or ethanol) and CO_2. Sulfate, acting as an electron acceptor, is reduced to generate H_2S. NO_3-reducing bacteria, containing nitrate reductase (such as the species in the family *Enterobacteriaceae*, some *Bacillus* spp., and *Staphylococcus* spp.), degrade metabolizable carbohydrates through EMP, HMS, and ED (also mixed acid fermentation) pathways. Pyruvate produced through these pathways can act as an effective electron donor and, depending on the species, may be converted to lactate, acetate, ethanol, formate, CO_2, H_2, butanediol, acetoin, and succinate.

F. Aerobic Respiration

Aerobes (*Bacillus* spp., *Pseudomonas* spp., molds, and yeasts) and many facultative anaerobes (*Enterobacteriaceae, Staphylococcus* spp.) under aerobic conditions can use molecular oxygen as the terminal electron acceptor during metabolism of carbohydrates to produce pyruvate by one or more of the major pathways mentioned earlier. Pyruvate, as well as other carboxylic acids, can be oxidized completely through oxidative decarboxylation to generate CO_2, H_2O, and large quantities of ATP. The pathway (designed as the Krebs cycle, the tricarboxylic acid cycle, or the citric acid cycle) also generates large numbers of intermediates that are utilized to synthesize cell materials. Initially, pyruvate is decarboxylated to generate acetyl ~ CoA and CO_2. Acetyl ~ CoA then combines with oxaloacetate (4C compound) to produce citrate (6C compound). Through successive reactions, citrate is metabolized to a 5C compound (and CO_2) and 4C compounds (and CO_2). The 4C succinate is then, through several steps, converted to oxalacetate for reuse. During these reactions, reducing compounds are generated that, in turn, enter the electron transport system, thereby generating $2H^+$ and $2e^-$. The terminal cytochrome, cytochrome oxidase (cyt.a), releases the electron for its acceptance by oxygen. If the cyt.a transfers only two pairs of electrons to molecular oxygen, the end product is H_2O; if one pair of electrons is transferred, the product is H_2O_2, which is subsequently hydrolyzed by microbial catalase or peroxidase to H_2O and O_2. Each pyruvate can potentially generate 15 ATP molecules.

G. Synthesis of Polymers

Leuconostoc mesenteroides cells growing on sucrose hydrolyze the molecules and predominantly metabolize fructose for energy production. Glucose molecules are polymerized to form dextran (polymer of glucose). Polymers are also formed from carbohydrates by some *Lactococcus lactis* and *Lactobacillus* strains, *Alcaligenes faecalis* strains, and *Xanthomonas* spp. Some of these polymers are useful as food stabilizers and to give viscosity in some fermented foods; they can also cause quality loss in some foods.

Metabolism of food carbohydrates by microorganisms is undesirable when it is associated with spoilage. On the other hand, fermentation of carbohydrates is desirable in food bioprocessing and production of metabolites for use in foods (such as lactate and diacetyl). Several end products are also used to identify microorganisms; for example, 2,3-butanediol production by *Enterobacter* spp. helps to differentiate them from the nonproducer *Esc. coli* strains (Voges Proskauer test). The microbial ability to metabolize different polysaccharides, disaccharides, and monosaccharides is also used to identify unknown isolates.

IV. METABOLISM OF FOOD PROTEINS

Proteinaceous compounds present in foods include different types of simple proteins (e.g., albumin, globulin, zein, keratin, and collagen), conjugated proteins (e.g., myoglobin, hemoglobin, and casein), and peptides containing two or more amino acids. Amino acids, urea, creatinine, trimethyl amine, and others form the nonprotein nitrogenous (NPN) group. In general, microorganisms can transport amino acids and small peptides (ca. 8 to 10 amino acids long) in the cells. Proteins and large peptides in a food are hydrolyzed to amino acids and small peptides by microbial extracellular proteinases and peptidases. Species from genera *Alcaligenes, Bacillus, Clostridium, Enterococcus, Enterobacter, Flavobacterium, Klebsiella, Lactococcus, Micrococcus, Pseudomonas*, and *Serratia* are among those capable of producing extracellular proteinases and peptidases. Small peptides are transported in the cell and converted to amino acids before being metabolized further.[1,4,5]

A. Aerobic Respiration (Decay)

Many aerobic and facultative anaerobic bacteria can oxidize amino acids and use them as their sole source of carbon, nitrogen, and energy. L-Amino acids generally undergo either oxidative deamination or transamination to produce respective keto acids, which are then utilized through different pathways. Several amino acids can also be oxidized in different pathways by many bacterial species. Some examples are conversion of L-threonine to acetaldehyde and glycine, L-tryptophan to anthranilic acid, L-lysine to glutaric acid, L-valine to ketoisovalerate, L-leucine to ketoisocaproate, L-arginine to citrulline, and L-histidine to urocanic acid.

B. Fermentation (Putrefaction)

Degradation of L-amino acids by anaerobic and facultative anaerobic bacteria is carried out either with single amino acids or two amino acids in pairs. Metabolism of single amino acids is carried out through different types of deamination (producing the C-skeletone and NH_3), decarboxylation (producing amines and CO_2), and hydrolysis (producing the C-skeletone, CO_2, NH_3, and H_2). The C-skeletones (fatty acids, α-keto acids, unsaturated acids) are then used to supply energy and other metabolic products. The metabolism of amino acids in pairs involves simultaneous oxidation–reduction reactions between suitable pairs in which one acts as the hydrogen

donor (oxidized) and the other as the hydrogen acceptor (reduced). Alanine, leucine, and valine can be oxidized, whereas glycine, proline, and arginine can be reduced by this type of reaction (Stickland reaction). The products in this reaction are fatty acids, NH_3, and CO_2.

The products of microbial degradation of amino acids vary greatly with the types of microorganisms and amino acids and the redox potential of the food. Some of the products are keto acids, fatty acids, H_2, CO_2, NH_3, H_2S, and amines. Metabolic products of several amino acids are of special significance in food because many of them are associated with spoilage (foul smell) and health hazards. They include indole and skatole from tryptophan; putrescine and cadaverine from lysine and arginine; histamine from histidine; tyramine from tyrosine; and sulfur-containing compounds (H_2S, mercaptans, sulfides) from cysteine and methionine. Some of these sulfur compounds, as well as proteolytic products of proteinases and peptidases (both extra- and endocellular) of starter-culture microorganisms are important for desirable and undesirable (bitter) flavor and texture in several cheeses. The breakdown of threonine to acetaldehyde by *Lactobacillus acidophilus* is used to produce the desirable flavor in acidophilus yogurt. Indole production from tryptophan is used to differentiate *Esc. coli* from other coliforms. Also, the amino acid metabolism profile is used in species identification of unknown bacterial isolates. In addition to degradation (catabolism) of proteinaceous compounds of foods, the synthesis (anabolism) of several proteins by some foodborne pathogens while growing in foods is important because of the ability to produce proteins that are toxins. They include thermostable toxins of *Staphylococcus aureus*, thermolabile toxins of *Clostridium botulinum*, and toxins produced by some bacteria associated with foodborne infections (such as Shiga toxin). The ability of some microbial species to synthesize essential amino acids (such as L-lysine), antibacterial peptides (such as nisin and pediocin), and enzymes (such as amylases and proteinases) in relatively large amounts has been used for beneficial purposes in foods.

V. METABOLISM OF FOOD LIPIDS

The main lipids in food are mono-, di-, and triglycerides; free saturated and unsaturated fatty acids; phospholipids; sterols; and waxes, with the glycerides being the major lipids. Microorganisms have low preference for metabolizing lipids. Being hydrophobic, lipids are difficult to degrade when present in a large mass. In emulsion, they can be metabolized by the microorganisms at the oil–water interphase. Glycerides are hydrolyzed by extracellular lipases to release glycerol and fatty acids. The fatty acids then can be transported inside the cells and metabolized by β-oxidation to initially generate acetyl ~ CoA units before being utilized further. Fatty acids, if produced at a rapid rate, accumulate in the food. Unsaturated fatty acids can be oxidized by microbial oxidases to initially produce hydroperoxides and then carbonyl compounds (aldehydes and ketones).

Some of the microorganisms that are important in food and can release lipases (hydrolytic enzymes) are found in the following genera: *Alcaligenes, Enterobacter, Flavobacterium, Micrococcus, Pseudomonas, Serratia, Staphylococcus, Aspergillus,*

Geotrichum, and Penicillium. Oxidative enzymes are produced mainly by the molds. Both groups of enzymes are associated with food spoilage, but oxidative enzymes are also important for desirable flavor in mold-ripened cheeses.

VI. CONCLUSION

Microbial growth in food is accomplished through the metabolism of food nutrients, principally the metabolizable carbohydrates, proteins, and lipids. Many metabolic pathways can be used, which are determined by the nature and concentration of a nutrient, the microbial type, and the oxidation–reduction potential of the food environment. Many types of end products are produced during metabolism of the nutrients, which, depending on the chemical nature, are associated with food spoilage, food poisoning, or production of fermented food. Some are also used to improve texture and flavor of foods. The metabolic end products are also useful to identify unknown microbial isolates from a food. As long as the physical and nutritional environments are maintained, bacterial cells continue to multiply, generating energy and cellular materials through the metabolic processes. However, a change in the environment can cause some species to shut down the cell multiplication cycle and trigger the sporulation cycle, in which a cell is differentiated as an endospore. This survival strategy under unfavorable conditions and its importance in food microbiology are discussed in Chapter 8.

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QUESTIONS

1. Discuss the major differences among aerobic respiration, anaerobic respiration, and fermentation of food nutrients by microorganisms.
2. Discuss how aerobes, anaerobes, and facultative anaerobes differ from each other in their ability to transfer electrons by different acceptors.
3. A facultative bacterial species is growing anaerobically in the following foods: one rich in carbohydrates but low in proteins, the second rich in proteins but low in carbohydrates, and the third rich in both. Also, the carbohydrates and proteins

in all three foods can be metabolized by the bacterial species. Suggest how the bacterial species will metabolize the two nutrients in their foods.

4. How are the polysaccharides and disaccharides metabolized by microorganisms?
5. List the five major pathways microorganisms use to metabolize monosaccharides found in foods.
6. List some metabolites produced by *Esc. coli* and *Enterobacter* spp. by mixed acid fermentation, and *Clostridium* spp. by butyric acid fermentation.
7. Discuss briefly the significance of microbial metabolism of carbohydrates by aerobic and anaerobic respiration.
8. How are food proteins metabolized by bacteria? What is the importance of protein synthesis during growth of microorganisms in food?
9. Discuss the differences and importance of amino acid degradation by microorganisms via fermentation and aerobic respiration in food.
10. What is the protein sparing effect and how can it be used advantageously in the production of low-carbohydrate, high-protein foods?
11. List three examples of metabolism of amino acids that can be used for laboratory identification of an unknown bacterial isolate from food.
12. What is the significance of lipid metabolism by microorganisms in food?

CHAPTER 8

Microbial Sporulation and Germination

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I. INTRODUCTION

Microorganisms important in food normally divide by binary fission (or elongation, as in nonseptate molds). In addition, molds, some yeasts, and some bacteria can form spores. In molds and yeasts, sporulation is associated with reproduction (and multiplication), whereas in bacteria it is a process of survival in an unfavorable environment. In molds and yeasts, sporulation can occur by sexual and asexual reproduction, and sexual reproduction provides a basis for strain improvement for those that are used industrially. In bacteria, sporulation occurs through differentiation and it provides a means to retain viability in a harsh environment. Among the spores, bacterial spores have special significance in foods, because of their resistance to many processing and preservation treatments used in food. Compared with bacterial spores, mold and yeast spores are less resistant to such treatments. Spore formation in molds, yeasts, and bacteria is briefly discussed here.

II. MOLD SPORES

Molds form spores by both asexual and sexual reproduction and on this basis are classified as perfect or imperfect molds, respectively. Molds form large numbers of asexual spores and, depending on the type, can form conidia, sporangiaspores, and arthrospores. Conidia are produced on special fertile hyphae called conidiophores (Figure 8.1). Among the important molds in food, *Aspergillus* and *Penicillium* species form conidia. Sporangiospores are formed in a sack (sporangium) at the tip of a fertile hypha (sporangiophores). *Mucor* and *Rhizopus* species are examples of molds that form sporangiospores. Arthrospores, formed by the segmentation of a hypha, are produced by *Geotrichum*. An asexual spore in a suitable environment germinates to form a hypha and resumes growth to produce the thallus. Sexual spores form from the union of the tips of two hyphae, two gametes, or two cells. However, among the molds important in food, sexual reproduction is rarely observed. Some examples include *Mucor* and *Neurospora*.

III. YEAST SPORES

On the basis of sporeforming ability, yeasts important in food are divided into two groups: those that can produce sexual ascospores are designated as *Ascomycetes* (true yeasts), and those that do not form spores are called false yeasts. Examples of some yeasts important in food that form ascospores are *Saccharomyces, Kluyveromyces,*

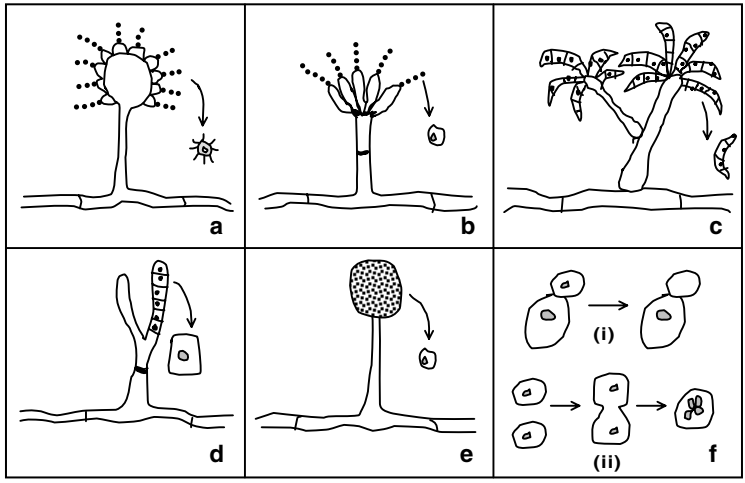


Figure 8.1 Schematic diagrams of spores of molds and yeasts. Conidiophore with condial head and condia (one magnified in each) of (a) *Aspergillus* spp., (b) *Penicillum* spp., and (c) *Fusarium* spp.; (d) arthrospore in *Geotrichum* spp.; (e) sporangiophore with sporangium containing sporangispores (one magnified) of *Rhizopus* spp.; (f) ascopore formation in yeasts by conjugation of (i) mother–daughter and (ii) two separate cells.

Pichia, and *Hansenula*. Species in the genera *Candida, Torulopsis*, and *Rhodotorula* do not form spores. Ascospores form by the conjugation of two yeast cells; in some cases, this can result from the union of the mother cell and a bud (daughter cell) (Figure 8.1). The number of spores developed in an ascus varies with species. In a suitable environment, each spore develops into a yeast cell.

IV. BACTERIAL SPORES

The ability to form spores is confined to only a few bacterial genera, namely the Gram-positive *Bacillus, Alicyclobacillus, Clostridium, Sporolactobacillus*, and *Sporosarcina* and the Gram-negative *Desulfotomaculum* species. Among these, *Bacillus, Alicyclobacillus, Clostridium*, and *Desulfotomaculum* are of considerable interest in food, because they include species implicated in food spoilage and foodborne diseases.[1–3] Several *Bacillus* and *Clostridium* species are used to produce enzymes important in food bioprocessing.

In contrast to mold and yeast spores, bacterial cells produce endospores (inside a cell) and one spore per cell. During sporulation and until a spore emerges following cell lysis, a spore can be located terminal, central, or off-center, causing bulging of the cell. Under a phase-contrast microscope, spores appear as refractile spheroid or oval structures. The surface of a spore is negatively charged and hydrophobic. Spores, as compared with vegetative cells, are much more resistant to physical and chemical antimicrobial treatments, many of which are employed in the processing and preservation of food. This is because the specific structure of bacterial spores is quite different from that of vegetative cells from which they are formed. From inside to outside, a spore has the following structures (Figure 8.2): a protoplasmic core containing important cellular components such as DNA, RNA, enzymes, dipicolinic acid (DPN), divalent cations, and very little water; an inner membrane, which is the forerunner of the cell cytoplasmic membrane; the germ cell wall, which surrounds this membrane, and is the forerunner of the cell wall in the emerging vegetative cell; the cortex, around the cell wall, composed of peptides, glycan, and an outer forespore membrane; and the spore coats, outside the cortex and membrane, composed of layers of proteins that provide resistance to the spores. Spores of some species can have a structure called exosporium outside the coat. During germination and outgrowth, the cortex is hydrolyzed, and outer forespore membrane and spore coats are removed by the emerging cell.

The spores are metabolically inactive or dormant, can remain in dormant form for years, but are capable of emerging as vegetative cells (one cell per spore) in a suitable environment. As opposed to nonsporeforming bacteria, the life cycle of sporeforming bacteria has a vegetative cycle (by binary fission) and a spore cycle (Figure 8.3). The spore cycle also goes through several stages in sequence, during which a cell sporulates and a vegetative cell emerges from a spore. These stages are genetically controlled and influenced by different environmental parameters and biochemical events, which are briefly discussed here.

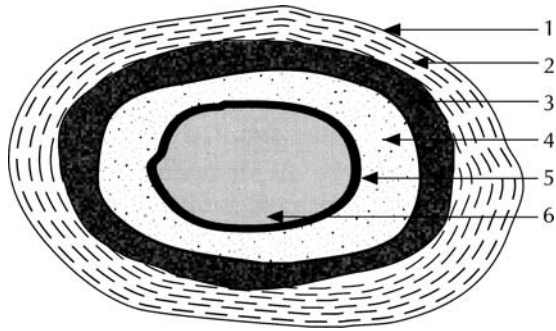


Figure 8.2 Schematic section of a bacterial spore showing different structures: (1) exosporium, (2) coat, (3) outer membrane, (4) cortex, (5) germ cell wall and inner membrane, and (6) core.

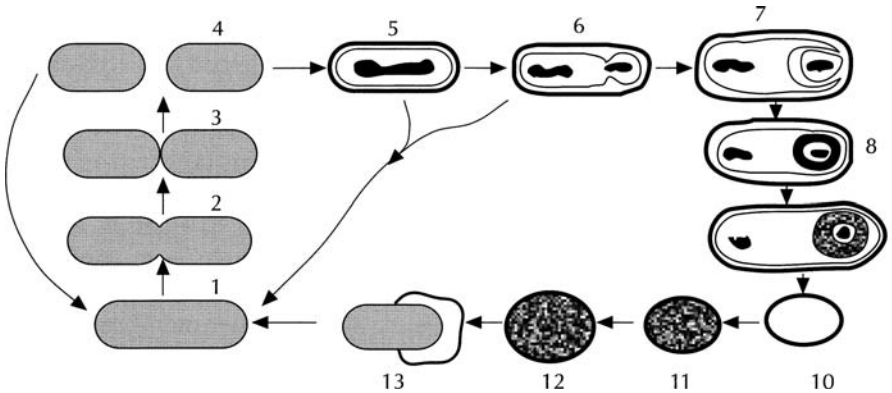


Figure 8.3 Schematic presentation of the cycles of cell multiplication (1–4) and endospore formation, germination, and outgrowth of sporeforming bacteria (5-13). Different steps are (5) formation of axil filament, (6) septation, (7) prespore formation, (8) cortex formation, (9) coat formation, (10) free spore, (11) germination following activation, (12) swelling of spore, (13) outgrowing cell. Cells from Step 4 can either divide (1–4) or sporulate. Cells from Step 5 and Step 6 can reverse back to cell division; from Step 7 the process is irreversible.

A. Sporulation

The transition from a normal vegetative cell cycle to sporulation in sporeforming bacteria is triggered by the changes in the environmental parameters in which the cells are growing as well as a high cell number in the environment. The environmental factors include reduction in nutrient availability (particularly carbon, nitrogen, and phosphorous sources) and changes in the optimum growth temperature and pH. Transition from cell division cycle to sporulation is genetically controlled, involving many genes. A cell initiates sporulation only at the end of completion of DNA replication. A triggering compound may be involved at that time for a cell to decide to either go through normal cell division or to initiate steps for sporulation. The triggering compound is probably synthesized when nutrition depletion and other

unfavorable conditions occur. Adenosine bistriphosphate (Abt) could be one of the triggering compounds, as it is synthesized by sporeformers under carbon or phosphorous depletion.

Sporulation events can be divided into about seven stages[1,2,4] (Figure 8.3):

1. Termination of DNA replication, alignment of chromosome in axial filament, and formation of mesosome
2. Invagination of cell membrane near one end and completion of septum
3. Engulfment of prespore or forespore
4. Formation of germ cell wall and cortex, accumulation of Ca^{2+}, and synthesis of DPN
5. Deposition of spore coats
6. Maturation of spore: dehydration of protoplast, resistance to heat, and refractile appearance
7. Enzymatic lysis of wall and liberation of spore

The process is reversible before Stage 3. However, once the process has entered Stage 3, a cell is committed to sporulation.[1,2]

B. Dormancy

Spores are formed in such a manner as to remain viable in unfavorable conditions. This is achieved by increasing their resistance to extreme environments and reducing metabolic activity to dormancy. Dehydration of the core and reduced molecular movement have been attributed to dormancy.

In a suitable environment, the dormancy of a spore can be ended through a series of biochemical reactions involved in spore activation, germination, outgrowth, and growth. Some spores may need a long time before they go through the sequences of germination, and are called superdormant spores. They are quite common in *Bacillus* and *Clostridium.* Superdormancy is thought to be the consequence of the inherent nature of a spore, spore injury, and environmental factors. Some spores have stringent germination needs and do not germinate with other spores. Injured spores need to repair their injury before they can germinate and outgrow. Some component in the media can prevent germination of some spores. In food, superdormant spores could cause problems. Following processing, they may not be detected in a food by conventional testing methods. But during storage, they can germinate and outgrow and subsequently cause spoilage of a food, or, if a pathogen, a spore can make a food unsafe for consumption.[1,2]

C. Activation

Spore activation before germination is accompanied by reorganization of macromolecules in the spores. Spores can be activated in different ways, such as sublethal heat treatment, radiation, high pressure treatment with oxidizing or reducing agents, exposure to extreme pH, treatment with high pressure, and sonication. These treatments probably accelerate the germination process by increasing the permeability of spore structures to germinating agents for macromolecular reorganization. This

process is reversible, i.e., a spore does not have to germinate after activation if the environment is not suitable.[1,2]

D. Germination

Several structural and functional events occur during germination. Once the germination process starts, the dormant stage is irreversibly terminated. Structural changes involve hydration of core, excretion of Ca^{2+} and DPN, and loss of resistance and refractile property. Functional changes include initiation of metabolic activity, activation of specific proteases and cortex-lytic enzymes, and release of cortex-lytic products. Generally, germination is a metabolically degradative process.

Germination can be initiated (triggered) by low pH, high temperature, high pressure, lysozyme, nutrients (amino acids, carbohydrates), calcium-DPN, and other factors. The process can be inhibited by D-alanine, ethanol, EDTA, NaCl (high concentrations), NO_2, and sorbate.[1,2]

E. Outgrowth

Outgrowth constitutes the biosynthetic and repair processes between the periods following germination of a spore and before the growth of a vegetative cell. The events during this phase include swelling of the spore due to hydration and nutrient uptake; repair and synthesis of RNA, proteins, and materials for membrane and cell wall; dissolution of coats; cell elongation; and DNA replication. The factors that can enhance the process include favorable nutrients, pH, and temperature. With the termination of the outgrowth stage, vegetative cells emerge from spores and enter the vegetative cell cycle of growth by binary fission.[1,2,4]

The sporulation process, as discussed previously, involves irreversible cellular differentiation processes in an unfavorable environment and is regulated and expressed by a large number of genes. The functions of many genes have been studied with mutants of *Bacillus subtilis* (*spo* mutants) that block formation of different components during the sporulation process. Expression of these functional genes is regulated by the genes coding for specific sigma factors (δ). In a favorable environment, germination and outgrowth are also mediated through other genes that enable an endospore to enter in the cell division cycle.[1]

V. IMPORTANCE OF SPORES IN FOOD

Spore formation, especially by molds and some bacterial species, enables them to survive for a long time and provides a basis for the continuation of the species. It also provides a means of their easy dissemination by dust and air in the environment.[1] In this manner, foods can be contaminated by their spores rather easily from various sources. In a suitable food environment, spores germinate, grow, and produce undesirable (or desirable) effects. Mold and yeast spores are relatively sensitive to heat, and their growth can also be prevented by storing foods in the absence of air.

Many species of *Bacillus, Clostridium*, and *Desulfotomaculum* are associated with food spoilage and foodborne diseases. Due to high heat resistance, the spores are of special interest and importance in food processing. Special attention must be given to processing and preserving the foods so that the spores are either destroyed or prevented from undergoing germination and outgrowth, because ungerminated spores cannot cause spoilage or foodborne disease. Another possibility is to induce the spores to germinate and outgrow and then expose them to an antibacterial treatment to destroy them. Superdormant spores of the spoilage and pathogenic species pose another problem. As they are not detected normally with the other spores, a processing condition could be wrongly adopted with the idea that it will eliminate all spores. Subsequently, these surviving superdormant spores can germinate, outgrow, and grow, and either cause the food to spoil or make it unsafe. As it is impossible to destroy all spores in many foods, several specific methods or a combination of processing and preservation methods have been developed to overcome problems of spores in food.[1,2]

In the canning of low-acid food, very high heat treatment is employed to achieve commercial sterility that kills spores of all pathogenic bacteria and most spoilage bacteria (except some of thermophilic spoilage bacteria). To prevent germination of spores, depending on the food type, nitrite (in processed meat), low pH (acid products), low A_w, or high salt are used. High hydrostatic pressure is currently being studied to determine its spore destruction potential. Although spores of molds are destroyed at relatively low pressure (≤400 MPa), spores of many pathogenic and spoilage bacteria need a combination of very high pressure (≥700 MPa) and high temperature (≥90°C) to obtain commercial sterility.[5] It has been known for long that spores of many foodborne bacteria can be activated to germination and outgrowth at a lower pressure range. Following such a pressure treatment, another antibacterial treatment, such as another pressure cycle, heat, or antimicrobial preservative can be given to destroy the germinated and outgrown spores before cell growth starts. The influence of several parameters on germination, such as pressure range, time of pressurization, pressurization temperature, and holding time following pressurization, is currently being studied.[6] Table 8.1 presents the results of a study. It can be seen that under any given condition, germination induction varies greatly with species. In general, germination increases with pressure and temperature within the range studied. More such studies with many strains of the important species will provide more meaningful data to determine the potential of such treatment to control bacterial spores in food.

VI. CONCLUSION

Spore formation by certain yeasts, molds, and bacterial species is a means of survival and continuation of the life process. In yeasts and molds, sporulation occurs by asexual and sexual process; in bacteria, it occurs through differentiation, regulated and expressed by many genes.

Spore formation enables the dissemination of the species widely in the environment as well as contamination of foods. Their growth in food can be undesirable

Table 8.1 Germination Induction of Bacterial Endospores by Hydrostatic Pressure at 25 and 50°C

Bacterial Strain	Pressurization for 5 min at Temperature (°C)	% Germination Induction[a] at MPa[b]		
		138	345	483
Bacillus cereus ATCC 10876	25	99	99	99
	50	99	99	99
Bacillus stearothermophilus ATCC 12980	25	45	88	88
	50	99	98	98
Clostridium sporogenes PA 3679	25	0	0	4
	50	40	50	82
Clostridium perfringens ATCC 1027	25	17	21	29
	50	12	40	44

[a] The spore suspensions were pressurized for 5 min either at 25 or 50°C, then stored at 4°C for 1 h and heated at 75°C for 15 min to destroy the germinated spores. Germination induction was determined by enumerating the surviving spores and subtracting the number from the unpressurized (control) spore suspensions.
[b] MPa: MegaPascal (1 MPa = 145 psi)

when they cause spoilage and produce toxins (except yeasts) in food and can be desirable in the processing of some foods. Methods of destruction of spores and inhibition of germination of spores (of bacteria) by different means are used to control their growth in food.

Some bacterial species sporulate as a means of survival strategy under conditions of physical, chemical, or environmental stresses by genetically regulated processes. Cells of many bacterial species, when exposed to conditions suboptimal for growth or sublethal, manifest different characteristics, the control mechanisms of which are not properly understood currently. These conditions are observed with many food-borne bacteria and have important implications in food microbiology, which are discussed in Chapter 9.

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QUESTIONS

1. List the differences among mold, yeast, and bacterial spores.
2. List five genera of foodborne bacteria that form spores.
3. Draw and label the structure of a bacterial spore and discuss the functions or characteristics of each structural component.
4. List the stages between the formation of a bacterial spore and its emergence as a vegetative cell. Also, list the major events that occur in each stage.
5. Discuss the triggering mechanisms in sporulation and spore germination in bacteria.
6. Discuss the importance of bacterial spores in food.
7. Briefly discuss the methods used to control problems associated with bacterial spores in food.
8. Explain how low-range hydrostatic pressure can be combined with other antibacterial treatment to destroy bacterial spores in food.

CHAPTER 9

Microbial Stress Response in the Food Environment

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I. INTRODUCTION

Foodborne bacterial cells (and other microbes) are usually exposed to different physical and chemical environments during production, processing, preservation, storage, transportation, and consumption of foods as well as during microbiological quality evaluation of foods and food ingredients by the recommended procedures. As a consequence, bacterial cells may become stressed and manifest several types of altered characteristics. A stressed environment can be both in the suboptimal growth range and beyond the growth range (Figure 9.1). Depending on the nature and level of a stress, cells in a population can develop a higher level of resistance to the same as well as several other types of stresses, or suffer reversible sublethal injury or even apparently lose culturability (ability to multiply) in some recommended bacteriological media and methods or lose viability (or ability to multiply) permanently. Researchers have used many different terminologies for these altered states of bacterial cells, some of which are not well defined or are for the same condition or even scientifically contradictory. This has generated confusion among interested individuals and controversy among researchers. Currently, there is a move among some scientists researching in the area of microbial stress to conduct direct basic studies to understand the response of bacterial cells under different levels and the nature of stresses and to develop consensus scientific opinion for suitable definitions and specific terminologies.

In the last 50 years, three different broad terminologies have appeared in microbiology literature to describe altered characteristics of bacterial cells following exposure to some physical and chemical stresses: sublethal injury (in the 1960s to 1980s),[1–3] viable-but-nonculturable state (in the 1980s and 1990s),[4] and stress adaptation (in the 1990s)[5]. In most publications, each aspect has been treated as a separate and unique phenomenon. In this chapter, the three have been described separately, and then it is suggested that all three are probably related, differing only in the degree of bacterial response following exposure to different levels of a stress. Several edited books have been published in each of the three areas.[1–5]

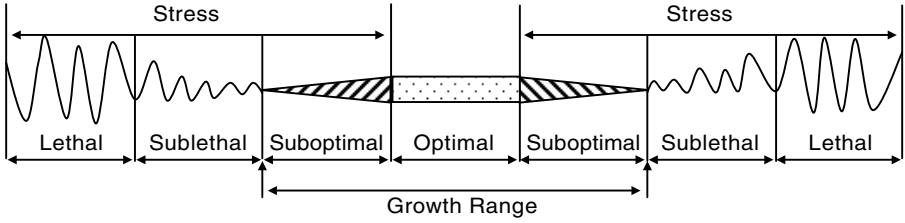


Figure 9.1 Different levels or degrees of environmental stresses to which bacterial cells can be exposed during processing and preservation of food. Bacterial cells exposed to a suboptimal growth condition show stress adaptation. Beyond the growth range, the cells are usually either sublethally or lethally stressed. See text for further explanations.

II. STRESS ADAPTATION

A. Definition and Observations

Stress adaptation or stress response has been explained as a situation whereby a brief exposure of a bacterial population to a suboptimal physical or chemical (growth) environment enables the cells to resist subsequent exposure to the same or other types of harsher treatment to which the species is normally susceptible. This phenomenon has been observed among many foodborne pathogens and spoilage bacteria following exposure of cells to various suboptimal physical and chemical environments, such as cold and warm temperature, low A_W, low hydrostatic pressure, UV light, high salt concentrations, bacteriocins, preservatives, detergents, several dyes, and antibiotics. It is assumed that a brief exposure to a suboptimal environment triggers some cellular mechanisms that enables them to resist subsequent exposure to harsher treatment.[5] However, once the cells are removed and allowed to grow for several generations in the optimum conditions, they do not remain resistant; rather, they revert to the original state. Several confusing and ambiguous terms have been used by different research groups to describe the stress adaptation phenomenon in bacteria. Some terms for suboptimal pH are included here.[6–8]

- *Acid Resistance or Acid Adaptation.* An exposure of cells for an extended period to mild acidic environment (e.g., pH 5.0 to 5.8), enables them to develop resistance to subsequent exposure to pH ≤ 2.5.
- *Acid Tolerance or Acid Tolerance Response (ATR).* A brief exposure of cells to mild acidic environment enables them to survive subsequent exposure to pH 2.4 to 4.0.
- *Acid Shock Response (ASR).* The response of bacterial cells to a low pH without previous adaptations to a mild pH.

Many studies have been conducted to determine stress adaptation of foodborne bacteria. *Escherichia coli* cells exposed for one or two generations at pH 5.0 survive better subsequently at pH 3 to 4 (but not pH <2). Similarly, a brief exposure of *Esc. coli* cells to 50°C enables the cells to survive better at 60°C (but not >72°C).[6,7] Similarly, acid-adapted *Listeria monocytogenes* cells survive well when exposed to pH 3.5. Acid-adapted *Lis. monocytogenes* cells also develop resistance to nisin. *Lis. monocytogenes* cells briefly exposed to 0.1% H_2O_2 also developed cross resistance against subsequent exposure to 0.5% H_2O_2, 5% ethanol, 7% NaCl, pH 5.0, or 45°C as compared with unadapted control cells.[8] Heat resistance (increase in *D*-value) of *Lis. monocytogenes*, *Salmonella* serovars, and *Esc. coli* O157:H7 suspended in low-pH fruit juices has been recently reported.[9] The cells of the pathogens were initially acid adapted by exposing to pH 5.0. They were then suspended in orange, apple, or grape juice (pH 3.5 to 3.9) and heated at 56°C. In all instances, the *D*-values of acid-adapted cells as compared with those of control cells increased significantly. For *Lis. monocytogenes*, *D*-values for control and acid-adapted cells were 2.1 and 3.8 min in orange juice, 1.6 and 5.0 min in apple juice, and 2.3 and 4.6 min in grape juice, respectively. These results suggest that stress adaptation of microorganisms can occur both in culture broth and in food systems. In food processing and

preservation methods, results of studies developed with normal cells (not stress adapted) may not be effective to control or kill stress-adapted foodborne pathogens and spoilage bacteria. To overcome this problem, it is necessary to understand the underlying mechanisms that confer resistance to stress-adapted cells and develop methods to control them.[10]

B. Mechanisms of Stress Adaptation[10,11]

An earlier theory was that bacterial cells cope with stress (such as temperature stress) by changing the lipid composition of the cytoplasmic or inner membrane so that the fluid state is maintained. Thus, at lower or higher ranges of growth temperature, membrane lipid accumulates more low-molecular-weight and unsaturated fatty acids but at optimum growth temperature accumulates more high-molecular-weight saturated fatty acids. In recent years, stress adaptation by bacterial cells (and other microorganisms) is viewed to be mediated through the synthesis of many types of shock proteins or stress proteins, some of which are specific for specific stress whereas others are nonspecific and expressed against more than one stress. Stress proteins provide protection to structures that could be otherwise adversely affected by the stress, such as DNA and many enzymes. Synthesis of stress proteins in large quantities is mediated through the expression of stress-related gene systems, some of which are inducible whereas others are constitutive but expressed at a low level when cells are not under stress. As some of the gene systems are global, gene expression by one stress can also help cells to adapt to other related stresses.

Expression of stress-related genes is initiated by specific polypeptides or sigma factor (δ) synthesized by specific genes. Some of these, such as δ^B or δ^{37} (encoded by gene *sig B*), help cope with general stress in Gram-positive bacteria; δ^{32} (encoded by *rpoH* gene) and δ^{24} (encoded by *rpoE* gene) help cope with heat response; and δ^{38} (encoded by *rpoS* gene) helps cope with general stress and starvation in Gram-negative bacteria. Under a specific stress (such as under heat-shock condition), *rpoH* is turned on to effect synthesis of Rpo *H* or δ^{32} protein in high amounts (Figure 9.2). This sigma factor (also called a regulon) then combines with the core RNA polymerase (consisting of four subunits, $\alpha\alpha\beta\beta'$) to form the complete RNA polymerase enzyme or holoenzyme. This holoenzyme then binds to the promoter of a heat-shock gene family, leading to synthesis of heat-shock proteins (e.g., in *Esc. coli*), which then protect the structural and functional units of stressed cells susceptible to heat damage (e.g., DNA and proteins). They can also protect against other stresses. Involvement of different sigma factors in protecting against stresses (such as cold, heat, low pH, UV) has been studied with several species of foodborne bacteria.

C. Importance of Stress-Adapted Microorganisms in Food[5,11]

As indicated before, during the handling of food and food ingredients from the farm to table, foodborne bacteria are exposed to different suboptimal physical and chemical environments. This can enable foodborne pathogens and spoilage bacteria, as well as beneficial bacteria, to develop characteristics that are different from those

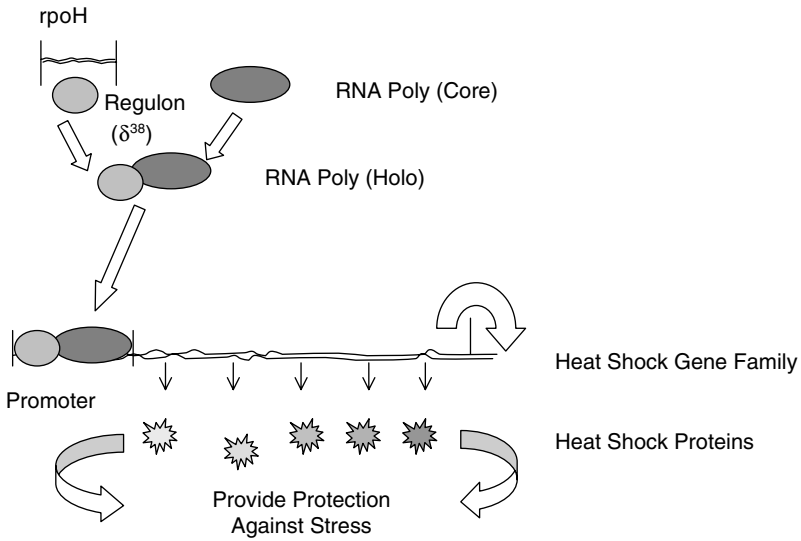


Figure 9.2 Genetic basis of coping stress with sigma factors by bacterial cells. See text for explanations.

of normal cells. This situation can be both disadvantageous and advantageous, as described next.

1. Pathogens and Spoilage Bacteria Surviving in Low-pH Foods

Most foodborne pathogens (especially the enteric pathogens) and spoilage bacteria (especially Gram-negative) are susceptible to low pH and die off rapidly in high-acid foods (pH ≤ 4.5) during storage. Also, at low pH, normal cells are susceptible to other antimicrobial treatments (such as hydrostatic pressure, pasteurization temperature, or preservatives) at a much lower level. However, if they are first acid-adapted, they become relatively resistant to lower pH and other treatments at minimal levels and survive in food. Recent occurrence of foodborne diseases from the consumption of fruit juices, fermented sausages, and acidified foods containing viable *Salmonella, Esc. coli* O157:H7, and *Lis. monocytogenes* are thought to be due to acid-adapted pathogenic strains surviving low pH and low heat treatment.

To overcome the problem, it is necessary to avoid exposure of the food (containing the target cells) to mild treatments; instead, several mild treatments can be used simultaneously to destroy the strains (hurdle concept, see Chapter 40).

2. Stress-Adapted Pathogens Surviving Stomach pH

Cells of most enteric pathogens associated with foodborne infections (see Chapter 25) are highly susceptible to low pH of the stomach and die off. This is probably one of the reasons why dose level is high ($\geq 10^6$ viable cells) for many enteric pathogens to cause infection in the gastrointestinal (GI) tract. Many of the cells

ingested are killed in the stomach, but a few survive, enter the GI tract, and set up infection. However, if a pathogenic strain in a food is stress adapted, even consumption of a much lower number will enable it to survive in the stomach and cause infection in the GI tract. An attempt should be made to eliminate or reduce the presence of stress-adapted pathogens in a ready-to-eat food.

3. Enhancing Viability of Starter Cultures and Probiotic Bacteria

Commercial starter cultures are normally frozen or freeze-dried before their use by the food processors with an intention of having high levels of survivors. Viability of these cultures, especially freeze-dried cultures, is generally low. Similarly, many probiotic bacteria are normally susceptible to stomach pH and low-pH food products (e.g., yogurt containing *Lactobacillus acidophilus*). However, first exposing the cultures to a mild stress to release stress proteins may enable the cells to survive subsequent freezing, freeze-drying, or exposure to low pH in the stomach or in food products. Genetic engineering techniques can also be used to develop new strains capable of producing cryoproteins and different stress proteins and surviving better.

III. SUBLETHAL STRESS AND INJURY

A. Definition and Observations

Sublethal injury occurs following exposure of bacterial cells to unfavorable physical and chemical environments (beyond the growth range but not in the lethal range) that cause reversible alterations in the functional and structural organizations of the cells.[1–3] As early as 1932, Rahn[12] suggested that death of microbes exposed to sublethal stresses is a gradual process that can be reversed under proper conditions if the reactions have not progressed too far. In 1959, Strake and Stokes[13] showed that bacterial cells exposed to cold temperature developed characteristics that were different from those of the normal population; the cells also temporarily lost the ability to multiply. However, in an appropriate environment, they repaired their injury and initiated multiplication. Later studies revealed that cells of yeasts and molds and spores of bacteria also incur reversible injury following exposure to sublethal stresses.

Many of the treatments include those used directly during food processing and storage as well as microbial detection from foods. Treatments include low heat (such as pasteurization), low temperature (freezing, refrigeration, and chilling), low A_w (different types of drying or adding high solutes such as sugar or salt), radiation (UV or x-ray), high hydrostatic pressure, electric pulse, low pH (both organic and inorganic acids), preservatives (sorbates or benzoates), sanitizers (chlorine or quaternary ammonium compounds), hot microbiological media (especially selective agar media above 48°C), and different selective enumeration and detection methods. This phenomenon is observed in many species of bacterial cells and spores, yeasts, and molds that are important in foodborne diseases, food spoilage, and food bioprocessing, and as indicators of sanitation (Table 9.1). From this list it becomes

Table 9.1 Cells and Spores of Some Microorganisms Important in Food in Which Sublethal Injury is Detected

Gram-positive pathogenic bacteria: *Sta. aureus, Clo. botulinum, Lis. monocytogenes, Clo. perfringens, Bac. cereus*
Gram-negative pathogenic bacteria: *Salmonella, Shigella* spp., enteropathogenic *Esc. coli*, *Esc. coli* O157:H7, *Vib. parahaemolyticus, Cam. jejuni, Yer. enterocolitica, Aer. hydrophila*
Gram-positive spoilage bacteria: *Clo. sporogenes, Clo. bifermentum, Bac. subtilis, Bac. stearothermophilus, Bac. megaterium, Bac. coagulans*
Gram-negative spoilage bacteria: *Pseudomonas* spp., *Serratia* spp.
Gram-positive bacteria used in food bioprocessing: *Lac. lactis* spp., *Lab. delbrueckii* subsp. *bulgaricus*, *Lab. acidophilus*
Gram-positive indicator bacteria: *Ent. faecalis*
Gram-negative indicator bacteria: *Esc. coli, Ent. aerogenes, Klebsiella* spp.
Bacterial spores: *Clo. botulinum, Clo. perfringens, Clo. bifermentum, Clo. sporogenes, Bac. cereus, Bac. subtilis, Bac. stearothermophilus, Des. nigrificans*
Yeasts and molds: *Sac. cerevisiae, Candida* spp., *Asp. flavus*

apparent that other microorganisms that have not been studied will most likely also be injured by sublethal stresses. In general, Gram-negative bacteria are more susceptible to injury than Gram-positive bacteria, and bacterial spores are much more resistant than vegetative cells to a particular stress.[1–3]

Microbial injury and growth of injured cells have been studied with both bacterial cells and spores. The material discussed here mainly covers bacterial cell and spore injury.

B. Manifestation of Bacterial Sublethal Injury

A bacterial population, following exposure to a sublethal stress, contains three physiologically different subpopulations: the uninjured (normal) cells, reversibly injured (injured) cells, and irreversibly injured (dead) cells. Their relative percentages vary greatly and are dependent on the species and strains, nature of suspending media, nature and duration of stress, and methods of detection. Injured cells differ in the level of injury and in several characteristics from their normal counterparts. One of these is increased sensitivity to many compounds to which normal cells are resistant, such as surface-active compounds (bile salts, deoxycholate, or SDS), NaCl, some chemicals (LiCl, selenite, bismuthsulfite, or tetrathionate), hydrolytic enzymes (lysozyme or RNase), antibiotics, dyes (Crystal Violet or Brilliant Green), and low pH and undissociated acids. They also lose cellular materials, such as K^+, peptides, amino acids, and RNA. When exposed to or maintained in an unfavorable environment, the injured cells die progressively. Also, the injured cells do not multiply unless the injury has been repaired. In a nonselective and preferably nutritionally balanced medium, the cells are able to repair their injury, which can extend 1 to 6 h, depending on the nature of stress and degree of injury. After an extended repair phase, the cells regain their normal characteristics and initiate multiplication. The injured cells in a population can be detected by a suitable nonselective enumeration technique. The results in Table 9.2 show that normal *Esc. coli* cells grow almost equally in both the nonselective and selective media, indicating that the cells are not sensitive to the surface-active agent, deoxycholate, in the medium. Following freezing and thawing,

Table 9.2 Effect of Freezing and Thawing on Viability Loss and Injury of *Esc. coli* NCSM

Enumeration Media[a]	Media	CFUs/ml		Subpopulations
		Before Freezing	After Freezing	
TS agar	Nonselective	276×10^6	17.5×10^6	Original population — dead: 276×10^6–17.5×10^6 (93.7%)
TSD agar	Selective	267×10^6	3.5×10^6	Among survivors — uninjured: 3.5×10^6 (19.9%); injured: $17.6 \times 10^6 - 3.5 \times 10^6$ (80.1%)

[a]*Esc. coli* cells in water suspension were enumerated before and after freezing (–20°C for 16 h) and thawing simultaneously in TS (tryptic soy) agar and TSD (TS + 0.075% deoxycholate) agar media by pour plating followed by incubation at 37°C for 24 h.

93.7% of cells die. The survivors form colonies in the nonselective TS agar (they repair and multiply), but 80.1% of the survivors fail to form colonies in the selective TSD agar (because of their injury and developed sensitivity to deoxycholate). Among the survivors, however, 19.9% are normal or uninjured cells, inasmuch as they grow equally well both in the TS and TSD agar media and are no longer sensitive to deoxycholate. Many food systems can reduce both the cell death and injury incurred from a specific stress.

Bacterial spore injury has been observed following heating, UV and ionizing radiation, treatment by hydrostatic pressure, and some chemicals (e.g., hypochlorite, H_2O_2, ethylene oxide, and probably nitrite) that are important in foods. The injured spores develop sensitivity to NaCl, low pH, NO_2, antibiotics, redox potential, gaseous atmosphere, and temperature of incubation. They also have delayed germination and longer lag for outgrowth, and they develop a need for some specific nutrients. These manifestations also vary with the nature of stress to which the spores are exposed.

C. Sites and Nature of Injury

Altered physiological characteristics of the injured bacterial cells have been used to determine directly the site of damage in the cellular structural and functional components. There is evidence that some cell components are damaged by almost all types of stresses studied. In addition, specific components can be damaged by specific stresses. The structural and functional components known to be damaged by sublethal stresses are the cell wall (or outer membrane, OM), cytoplasmic membrane (or inner membrane, IM), ribosomal RNA (rRNA) and DNA, and some enzymes (see Figure 2.2). Damages in the cell wall (or OM) and cytoplasmic membrane (or IM) are more evident in injury caused by freezing and drying, whereas damage to rRNA is more extensive in sublethal heating and DNA damage following radiation of cells.[1–3,14]

In Gram-positive and -negative bacteria, freezing and drying cause changes in cell surface hydrophobicity and the inability to form compact pellets and to adsorb some phages. In Gram-positive bacteria, surface layer proteins are also lost. In sublethally stressed Gram-negative bacteria, the lipopolysaccharide (LPS) layer undergoes conformational alteration and loses its barrier property to many chemicals (such as SDS, bile salts, antibiotics, lysozyme, and RNase), which can easily enter the injured cells and kill them. Very little LPS is lost from the cells into the environment. The alteration in conformation of LPS is due to loss of divalent cations, which are necessary for the normal stability of LPS. In both Gram-positive and -negative cells, the cytoplasmic membrane (or IM) remains intact in injured cells, but they lose their permeability barrier function. The cells become sensitive to NaCl and also lose different cellular materials. It is suggested that protein molecules in this structure probably undergo conformational changes in the injured cells. rRNA is extensively degraded by the activated RNase. DNA can undergo single- and double-strand breaks. In some strains, autolytic enzymes can be activated by stress, causing lysis of the cells.[1–3,14]

In bacterial spores, depending on the type of sublethal stress, different structural and functional components can be injured. High heat causes damage to the lytic enzymes necessary for the lysis of cortex before spore germination and to the spore membrane structures, causing loss of permeability barrier functions. Damages by irradiation (UV and γ) are mainly confined to DNA in the form of single-strand breaks, and by some chemicals (H_2O_2, antibiotics, or chlorine) to the lytic enzymes of the germination system. Hydrostatic pressure, in combination with heat, damages cortex, whereas γ-irradiation damages both cortex and DNA. Milder to strong acid treatments can cause the spores to become dormant by removing Ca^{2+} from the spores and making them sensitive to heat.[2,3]

D. Repair of Reversible Injury

One of the most important characteristics of injured bacterial cells is the ability to repair the injury in a suitable environment and become similar to normal cells. The repair process and the rate of repair can be measured by specific methods. One of them is to measure regain in resistance of injured cells to the surface-active agents by repair in the cell wall or the OM. Suspending a sublethally stressed population in a repair medium and simultaneously enumerating the colony-forming units (CFUs) during incubation in nonselective and selective plating media help determine the rate of repair (Figure 9.3). Initially, the injured survivors fail to form colonies in the selective, but not in the nonselective, media. However, as they repair and regain resistance to the selective agent, they form colonies on both media, as indicated by the increase in counts in the selective media only. Injured cells differ in the levels of injury (from low to high), as manifested from the differences in recovery time in a suitable medium.[1–3,14,15]

The injured cells can repair in a medium devoid of selective compounds but containing the necessary nutrients during incubation at optimum pH and temperature. In general, the cells repair well in a medium rich in metabolizable carbon and nitrogen sources and several vitamins. Supplementation with catalase and pyruvate

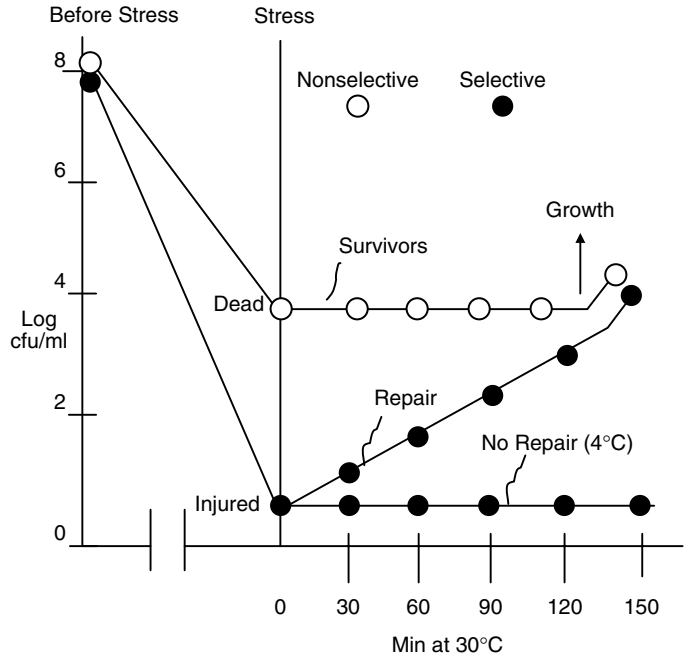


Figure 9.3 A hypothetical repair curve of injured bacteria. Repair is indicated by an increase in counts only in selective agar media during incubation in nonselective repair broth. Cell multiplication is indicated by a simultaneous increase in counts on both selective and nonselective agar media.

(to destroy H_2O_2 produced by the cells) also enhances repair and increases the number of repaired cells. It has been suggested that during the rapid repair process, many cells generate H_2O_2 but fail to hydrolyze it because of an injured peroxidase system. Accumulated H_2O_2 then causes cell death. A simple medium with a suitable energy source can also enable some cells with low level of injury to repair damage in the OM (or cell wall). Depending on the sublethal stress, complete repair can be achieved in 1 to 6 h at 25 to 37°C. For freezing and drying injuries, the rate is very rapid; for heat injuries, the rate can be slow. Specific studies show that the metabolic processes during repair vary with the nature of a stress and involve synthesis of ATP, RNA, DNA, and mucopeptide. Reorganization of the existing macromolecules can also be an important event during the repair process. The cell wall (or OM) regains the ability to prevent entrance of many chemicals to the cells; the cytoplasmic membrane (or IM) regains its permeability barrier function as well as the enzymes; and RNA and DNA regain their original characteristics. Finally, the cells regain their ability to multiply. Direct or indirect studies have not been conducted to show that the repair process is associated with activation or inactivation of genetic material.

Repair conditions for injured spores vary with the type (aerobic or anaerobic species) of spores. The composition of media is very important; in addition to good carbon and nitrogen sources, addition of special compounds, such as starch, a reducing compound (such as cysteine), lysozyme, and divalent cations, may be

necessary. Time, temperature, and gaseous environment also have to be optimal for the species. Generally, a longer repair time is required by spores damaged by heat, radiation, and chemicals than by low temperature.[1,14,15]

E. Injury in Yeasts and Molds

Very limited studies on injury and repair in yeasts and molds have been conducted. Freezing, low heat treatment, and irradiation have been reported to cause injury in vegetative cells of *Saccharomyces, Kluyveromyces, Candida, Aspergillus, Penicillium*, and *Rhizopus* spp. Spores of the molds have been found to be damaged by irradiation. The main characteristic of the injured cells and spores is their increased sensitivity to many selective environments. The cell membrane seems to be the major structure implicated in injury. Repair in nutritionally rich, nonselective media occurs before multiplication.[1–3]

F. Importance of Sublethally Injured Microorganisms in Food

Many of the physical and chemical treatments able to induce sublethal injury in microbial cells and spores are used in the processing, storage, and preservation of foods and the sanitation of facilities. Thus, it is quite likely that the foods and the facilities will harbor injured microorganisms. Microbial injury is important in food microbiology for several reasons.[14–16]

1. Detection of Undesirable Microorganisms

Injured microorganisms are potentially capable of multiplying. Thus, injured pathogens can cause foodborne disease, and injured spoilage microorganisms can reduce the shelf life of a product. It is important that they be detected if present in a food. For detection of many microorganisms in food, several types of selective liquid and solid media are used (Appendix E). Injured microorganisms may not be detected in these media. As a result, foods containing viable but injured pathogens and indicators above the regulatory or acceptable limits, and high numbers of spoilage microorganisms, can be sold. These products can be hazardous and have a short shelf life, despite meeting the regulatory standards and specifications. To overcome these problems, a short repair phase has been incorporated before the selective detection procedures of important microorganisms in foods. This information is also important in designing processing parameters to obtain proper reduction of undesirable microorganisms in finished products (such as heating temperature and time).

2. Enhancing Shelf Life of Foods

Injured cells are susceptible to many physical and chemical environments. These conditions, where possible, can be used in the preservation of foods (such as low temperature, lower pH, or preservatives for low-heat-processed foods) to kill injured cells and spores. In this manner, they will be unable to repair, and their potential ability to grow and cause product spoilage can be reduced.

3. Enhancing Viability of Starter Cultures

In the bioprocessing of foods, starter cultures are used as frozen concentrates or freeze-dried preparations. However, both these conditions are known to cause death and injury to the cells. By studying the mechanisms responsible for cell death or cell injury, it may be possible to stop the events and reduce death and injury. This will help produce starter concentrates that can be stored for a long time without a reduction in their viability or desirable characteristics.

IV. VIABLE-BUT-NONCULTURABLE

A. Definition and Terminologies

Under unfavorable environments, some cells in a bacterial population remain viable but are not able to multiply in many recommended bacteriological media, unless they are subjected to a prior resuscitation treatment. This phenomenon has been reported with starved cells of *Vibrio vulnificus, Vib. cholerae, Vib. parahaemolyticus, Salmonella* serovars*, Esc. coli* O157:H7, *Campylobacter jejuni, Enterococcus faecalis, Shigella dysenterie, Helicobacter pylori, Pseudomonas fluorescens*, and others exposed to low temperature, seawater, water, saliva, phosphate buffer, and salted salmon roe. Because this phenomenon is observed with many foodborne pathogens, a concern was raised that the pathogens can be present in a food in a viable-but-nonculturable (VBNC or VNC) state but could not be detected by the recommended methods. However, these cells can resuscitate under a favorable environment, multiply, and, following consumption of the food, cause foodborne diseases.[4,17,18]

In recent years, the existence of the VBNC state of bacterial cells has been questioned because of the study methods used by researchers. In addition, the scientific validity of the VBNC terminology, especially in food microbiology, has been questioned by others. The operational definition of some terminologies related to this phenomenon is listed here:[19,21]

- *Viability.* Cells that are metabolically active and able to multiply in an appropriate environment. In bacteriology, this term is analogous with the term *culturability.*
- *Metabolically Active.* Cells capable of carrying out at least some metabolic activity but may not necessarily multiply.
- *Nonculturability.* Inability to multiply under any conditions.
- *Dead Cells.* Cells unable to divide in an appropriate environment.
- *Resuscitation.* Metabolically active cells changed from a state of inability to multiply in one environment to a state of ability to multiply in another environment.

These definitions suggest the term *viable-but-nonculturable* may be contradictory. The cells included under this term could fail to multiply in one selective environment but multiply in another environment. Also, following resuscitation, they can multiply in the selective environment (some similarities with sublethally injured cells).

Because of current controversy among researchers studying the VBNC phenomenon of bacterial cells, the views and observation of the groups are summarized in this chapter.

B. Proponent Views

The existence of the VBNC phenomenon was determined initially in starving *Vib. vulmificus* cells kept in artificial seawater at 5°C and simultaneously determining during storage the total microscopic cell counts (TMCs) following staining with acridine orange; direct viable cell counts (TVCs) microscopically following exposure either to an inhibitor that enables cells to elongate but prevents cell division or to a tetrazolium salt that is metabolized to formazan (a fluorescence compound) by the metabolically active cells; and enumerating CFUs in a selective agar media.[17,18] Figure 9.4 presents the results of a typical study. In such a study, the difference between TMC and TVC is regarded as dead cells and that between TVCs and CFUs is considered as VBNC cells. Researchers hypothesize that under unfavorable environmental conditions, the VBNC cells (in the populations) enter a dormant state as a survival strategy. As in sporulation in bacterial cells, this is an inducible, programmed strategy and part of the life cycle of the cells. The cells remain metabolically active but stopped multiplication. There are also suggestions that the formation of the VBNC state occurs in two phases. In the initial state, the cells lose culturability but retain cellular integrity and nucleic acid structures. Gradually, the integrity of cells is lost and degradation of nucleic acid starts, leading to cell death.

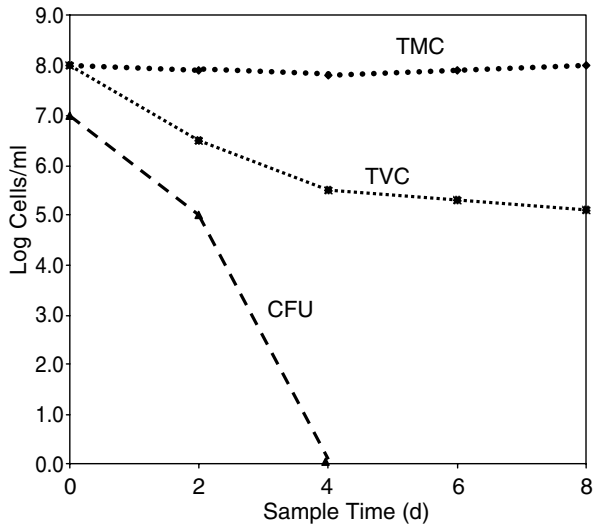


Figure 9.4 Total cell counts (TMCs), direct viable counts (TVCs), and colony forming unit (CFU) counts of a bacterial cell suspension in artificial seawater at 5°C. TMCs and TVCs are obtained microscopically and CFUs are obtained by enumeration on an agar medium. See text for other explanations. (Adapted from Colwell, R.R., *Zentbl. Bakteriol.*, 279, 154, 1993.)

Cells can be triggered to multiply by removing the VBNC-inducing factor or by supplying a resuscitation-inducing factor in the culture media. Also, heat shock, inoculation in egg embryo, exposure to HeLa cells, animal passage (for the pathogens), and other treatments can initiate multiplication (and restore disease-producing capability). Once resuscitated in an appropriate environment, the cells multiply rapidly like the cells in a normal population. Other indirect physiological studies have suggested that VBNC cells have increased cell volume and reduced internal K^+, membrane potential, membrane permeability, and adenylate energy (ATP). Structural DNA remains intact, respiratory activity is maintained, and expression of some genes is retained.

C. Opponent Views

Several questions have been raised by some researchers regarding the existence of the bacterial VBNC phenomenon. One is based on the hypothesis of the proponent group that the VBNC state is a survival strategy programmed in the life cycle and induced under unfavorable conditions (as in the sporulation process), and thus genetically determined.[22,23] Although the genetic basis of bacterial sporulation is well understood, there is no information to prove directly that the VBNC state in bacteria is programmed and has a genetic basis. This information is necessary to resolve the hypothesis. Another question has been raised on the study methods used to determine the VBNC phenomenon. In the direct method, VBNC cells in a population are determined from the differences in TVCs, determined microscopically, and CFUs, determined by agar media plating methods. Vital staining and optical methods used in TVC studies are not accepted by many in the scientific community as the effective methods to differentiate between dead cells and viable cells. In the indirect methods used to study cell volume, membrane permeability, membrane potential, the total population consisting of a mixture of dead cells (in large numbers), VBNC cells and cells capable of forming CFUs were used instead of using only the VBNC cells. Currently, no method is available to separate the three cell groups in a population and conduct studies on the cellular changes only in the VBNC cells. Therefore, the validity of these indirect studies is open to question. Finally, some have questioned the validity of the resuscitation method used by proponent groups to prove the existence of the VBNC phenomenon. An increase in CFUs following incubation of total population of stressed cells in a suitable environment for one or more days was suggested because of regain in culturability of the VBNC cells. Some researchers have suggested that this large increase in CFUs (as much as six or more log cycles) can be due to multiplication of a few viable cells during prolonged incubation. Determining simultaneously the TMCs, TVCs, and CFUs during resuscitation period, as was conducted during the determination of VBNC cells (Figure 9.4), would have proven that the increase in CFUs during resuscitation was not from growth of a few viable cells but from the transformation of VBNC cells to culturable state. Similarly, regain in pathogenicity of VBNC cells following resuscitation, as determined by the resuscitated cells to attach to HeLa cells, colonize egg embryo, and cause intestinal infection, has been suggested to result from the growth of a few viable cells in the original population.

D. Current Views

The results of several recent studies have provided a better insight of bacterial cells exposed to an unfavorable environment for a prolonged period. These results show that the so-called VBNC cells are really viable as well as culturable but are not recovered unless they are exposed to appropriate culture conditions.[24–27] Many of the starved or stressed cells have imbalanced metabolic pathways. When these cells are exposed to a nutritionally rich medium, they start metabolism and generate large quantities of superoxides and free radicals. However, because of defective metabolic processes, they cannot detoxify these products and are killed. This death can be prevented or greatly reduced by exposing the cells in a medium that either reduces the production of the superoxides and free radicals or increases their degradation. Supplementation of pyruvate or catalase to resuscitation media was thus found to transform the cells to a culturable state very rapidly, as determined by an almost immediate increase in CFUs without any change in TMCs. Several studies in the 1970s and 1980s on bacterial sublethal injury have shown that following environmental stress of a bacterial population, the recovery of the survivors can be increased either by incubating the cells in a minimal medium or in a nutritionally rich medium supplemented with pyruvate or catalase. It was suggested at that time that in a repair medium, especially in a nutritionally rich medium, the cells generate H_2O_2. The cells with greater levels or degrees of injury (see Figure 9.3) have defective peroxidase or catalase systems and fail to degrade H_2O_2; supplementation of a medium with pyruvate or catalse helps degrade the H_2O_2 and prevent cell death.[14,15]

It appears from the similarities of these observations that there are many similarities between the VBNC phenomenon and sublethal injury of bacterial cells. The differences are more in the study methods used and interpretation of the results.

E. Importance of VBNC Microorganisms in Food

The existence of bacterial cells that cannot be cultured by many currently recommended bacteriological methods cannot be disputed. They can be present in food and food environment and can potentially cause foodborne diseases and food spoilage. Food microbiologists have to recognize this and develop proper resuscitation and detection procedures for effective identification of their presence in food and safeguard the health of consumers and reduce waste of food from spoilage.

V. CONCLUSION

Bacterial cells exposed to environmental stresses respond differently, depending on the level or degree of a stress (Figure 9.5). In the suboptimal growth range, away from the optimal growth range, the cells can have a stress adaptation as manifested by an increased resistance to same and several other stresses at higher levels. An exposure beyond the growth range will cause injury in different structural and functional components of some cells without affecting some others. The levels or degrees of injury in the cells differ with the nature and duration of a stress, and

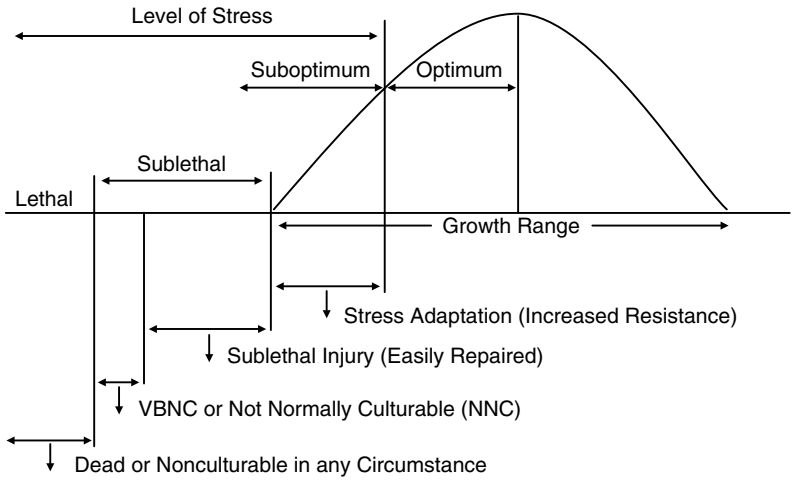


Figure 9.5 Schematic representation of bacterial stress response when exposed to a physical or chemical environment beyond optimum growth range. See text for explanations.

under any stress the level of injury increases as the level of stress increases. For some cells, the injury can be repaired rapidly in a nonselective or a nutritionally rich medium; these cells, usually designated as sublethally injured cells, probably have a low level of injury. Some other cells, probably because of high level of injury, require more exact resuscitation environment for repair; these cells probably include VBNC cells (alternatively designated as not normally culturable or NNC cells). Finally, some stressed cells fail to multiply under any resuscitation conditions and are designated as lethally injured (or dead) cells.

Foodborne pathogens and spoilage bacteria can be stress adapted, sublethally injured, or VBNC (or NNC) by the many processing, preservation, transportation, and storage conditions used in food production. They may not be detected from a contaminated food by many of the recommended methods used for their detection. Effective methods have to be developed and used for their detection in food and safeguard the health of the consumers and loss of food from spoilage.

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QUESTIONS

1. Define stress adaptation of foodborne bacterial cells and list six types of environments where bacterial cells can encounter stress during the handling of foods.
2. Describe the possible mechanisms by which bacterial cells can adapt to a suboptimal stress. Also explain how exposure to one stress can provide protection against multiple stresses.
3. List the importance of bacterial stress adaptation in food microbiology.
4. Explain the phenomenon of bacterial sublethal injury and list six conditions of food-handling procedures that can inflict sublethal injury to bacterial cells, bacterial spores, and yeast and molds.
5. List six altered characteristics (manifestations) of sublethally injured bacterial cells.
6. List two structural and two functional components of bacterial cells that show sublethal injury and indicate the nature of changes of the cell components.
7. Discuss the mechanism of repair of sublethal injury and the nature of repair environment.
8. A suspension of *Salmonella* cells was subjected to a sublethal stress. Enumeration of cell suspensions before and after stress and after repair in a nonselective broth for 1 h at 30°C provided the following data (CFUs/ml; NSA, nonselective agar; SA, selective agar media):

   Controlled cells — NSA: 3×10^7 and SA: 2.8×10^7
   Stressed cells — NSA: 0.5×10^7 and SA: 6×10^5
   Repaired cells — NSA: 0.4×10^7 and SA: 8×10^6

   Calculate the percentage of dead, injured, normal, repaired, and unrepaired cells.
9. List the importance of sublethal injury in food microbiology and explain how the phenomenon can be used in problem solving and for beneficial purposes.
10. Define the phenomenon of VBNC (or VNC) of bacterial cells. Explain the importance of the phenomenon.
11. Briefly list the findings and suggestions of the proponent groups on the VBNC phenomenon.
12. List the views of the opponent groups against the existence of the VBNC phenomenon.
13. Explain briefly the suggestions that could be associated with the so-called VBNC phenomenon in light of some recent findings.
14. How justified is the assumption that stress adaptation, sublethal injury, and VBNC state can be grouped together as different manifestations of bacterial cells under different levels or degrees of stress?

15. Define or explain the importance of the following terms (in food microbiology): stress protein; sigma factors; holoenzyme (RNA polymerase); loss of divalent cations of LPS layer; conformational alteration of structural and functional components under stress; spore injury; survival strategy of VBNC cells; culturability and nonculturability.

SECTION III

Beneficial Uses of Microorganisms in Food

The major concern of microbial presence in food is due to its undesirable properties. Most are able to spoil foods, and several are associated with foodborne health hazards. However, there are other microorganisms that have beneficial properties in food production, maintaining normal health of the gastrointestinal tract of humans and controlling the undesirable spoilage and pathogenic bacteria in food. The beneficial attributes of the desirable microorganisms are briefly discussed in this section through the following topics:

Chapter 10: Microorganisms Used in Food Fermentation
Chapter 11: Biochemistry of Some Beneficial Traits
Chapter 12: Genetics of Some Beneficial Traits
Chapter 13: Starter Cultures and Bacteriophages
Chapter 14: Microbiology of Fermented Food Production
Chapter 15: Intestinal Beneficial Bacteria
Chapter 16: Food Biopreservatives of Microbial Origin
Chapter 17: Food Ingredients and Enzymes of Microbial Origin

CHAPTER 10

Microorganisms Used in Food Fermentation

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I. INTRODUCTION

Beneficial microorganisms are used in foods in several ways. These include actively growing microbial cells, nongrowing microbial cells, and metabolic by-products and cellular components of microorganisms. An example of the use of growing microbial cells is the conversion of milk to yogurt by bacteria. Nongrowing cells of some

bacteria are used to increase shelf life of refrigerated raw milk or raw meat. Many by-products, such as lactic acid, acetic acid, some essential amino acids, and bacteriocins produced by different microorganisms, are used in many foods. Finally, microbial cellular components, such as single-cell proteins (SCPs), dextran, cellulose, and many enzymes, are used in food for different purposes. These microorganisms or their by-products or cellular components have to be safe, food grade, and approved by regulatory agencies. When the microbial cells are used in such a way that they are consumed live with the food (as in yogurt), it is very important that they and their metabolites have no detrimental effect on the health of the consumers. When a by-product (such as an amino acid) or a cellular component (such as an enzyme) is used in a food, the microorganisms producing it have to be regulated and approved, and the by-product and cellular component have to be safe. If a food-grade microorganism is genetically modified, its use in food has to be approved, especially if the genetic material used is obtained from a different source or is synthesized. Thus, the microorganisms used for these purposes have to meet some commercial and regulatory criteria. In this chapter, characteristics of some microorganisms used in the processing of foods (designated as fermented foods) are discussed. Many of these microorganisms are used to produce several by-products and cellular components used in foods.

II. MICROBIOLOGY OF FERMENTED FOODS

Food fermentation involves a process in which raw materials are converted to fermented foods by the growth and metabolic activities of the desirable microorganisms. The microorganisms utilize some components present in the raw materials as substrates to generate energy and cellular components, to increase in population, and to produce many usable by-products (also called end products) that are excreted in the environment. The unused components of the raw materials and the microbial by-products (and sometimes microbial cells) together constitute fermented foods. The raw materials can be milk, meat, fish, vegetables, fruits, cereal grains, seeds, and beans, fermented individually or in combination. Worldwide, more than 3500 types of fermented foods are produced. Many ethnic types are produced and used in small localities by small groups of people. Many of the fermented foods consumed currently have been produced and consumed by humans for thousands of years. The old city civilizations, dating as far back as 3000 to 5000 B.C. in the Indus Valley, Mesopotamia, and Egypt, developed exceptional skills in the production of fermented foods from milk, fruits, cereal grains, and vegetables. The process not only produced new foods but also helped preserve the excess of raw materials both of plant and animal origins.[1]

The basic principles developed by these ancient civilizations are used even today to produce many types of fermented foods by a process known as natural fermentation. In this method, either the desirable microbial population naturally present in the raw materials or some products containing the desirable microbes from a previous fermentation (called back slopping), are added to the raw materials. Then the fermentation conditions are set so as to favor growth of the desirable types but prevent

or retard growth of undesirable types that could be present in the raw materials. In another type of fermentation, called controlled or pure culture fermentation, the microorganisms associated with fermentation of a food are first purified from the food, identified, and maintained in the laboratory. When required for the fermentation of a specific food, the microbial species associated with this fermentation are grown in large volume in the laboratory and then added to the raw materials in very high numbers. Then the fermentation conditions are set such that these microorganisms grow preferentially to produce a desired product. Characteristics of some of the microorganisms used in fermentations are discussed here. These microbial species, when used in controlled fermentation, are also referred to as starter cultures. Many of these microbial species are present in raw materials that are naturally fermented, along with other associated microorganisms, some of which may contribute to the desirable characteristics of the products.

III. LACTIC STARTER CULTURES

At present, bacterial species from 12 genera are included in a group designated as lactic acid bacteria because of their ability to metabolize relatively large amounts of lactic acids from carbohydrates.[2–4] The genera include *Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Lactobacillus, Enterococcus, Aerococcus, Vagococcus, Tetragenococcus*, *Carnobacterium, Weissella,* and *Oenococcus*. Many of the genera have been created recently from previously existing genera and include one or a few species. For example, *Lactococcus* and *Enterococcus* were previously classified as *Streptococcus* Group N and Group D, respectively. *Vagococcus* is indistinguishable from *Lactococcus*, except that these bacteria are motile. *Weissella* and *Oenococcus* are separated from *Leoconostoc. Tetragenococcus* includes a single species that was previously included with *Pediococcus (Pediococcus halophilus). Carnobacterium* was created to include a few species that were previously in genus *Lactobacillus* and are obligatory heterofermentative. However, species from the first five genera, i.e., *Lactococcus, Leuconostoc, Pediococcus, Streptococcus,* and *Lactobacillus,* are used as starter cultures in food fermentation and are discussed here. The status of others, except *Tetragenococcus halophilus* and *Oenococcus oeni*, with respect to use in food, is not clear at present.

A. *Lactococcus*

This genus includes several species, but only one species, *Lactococcus lactic*, has been widely used in dairy fermentation. It has three subspecies (ssp.), ssp. *lactis*, ssp. *cremoris*, and ssp. *hordniae*, but only the first two are used in dairy fermentation. The biovar *Lac. lactis* ssp. *lactis* biovar diacetylactis is also used in dairy fermentation.[2,3]

The cells are ovoid, ca. 0.5 to 1.0 μm in diameter, present in pairs or short chains, nonmotile, nonsporulating, and facultative anaerobic to microaerophilic (Figure 10.1). In general, they grow well between 20 and 30°C, but do not grow in 6.5% NaCl or at pH 9.6. In a suitable broth they can produce ca. 1% L(+)-lactic acid and

reduce the pH to ca. 4.5. Subsp. *cremoris* can be differentiated from subsp. *lactis* by its inability to grow at 40ºC, in 4% NaCl, ferment ribose, and hydrolyze arginine to produce NH_3. Biovar diacetylactis, as compared with others, produces larger amounts of CO_2 and diacetyl from citrate. They are generally capable of hydrolyzing lactose and casein. They also ferment galactose, sucrose, and maltose. Natural habitats are green vegetation, silage, the diary environment, and raw milk.

B. *Streptococcus*

Only one species, *Streptococcus thermophilus*, has been used in dairy fermentation. A change in designation to *Str. salivarius* ssp. *thermophilus* was suggested but not made. They are used in dairy fermentation. The Gram-positive cells are spherical to ovoid, 0.7 to 0.9 μm in diameter, and exist in pairs to long chains (Figure 10.1). The cells grow well at 37 to 40ºC, but can also grow at 52ºC. They are facultative

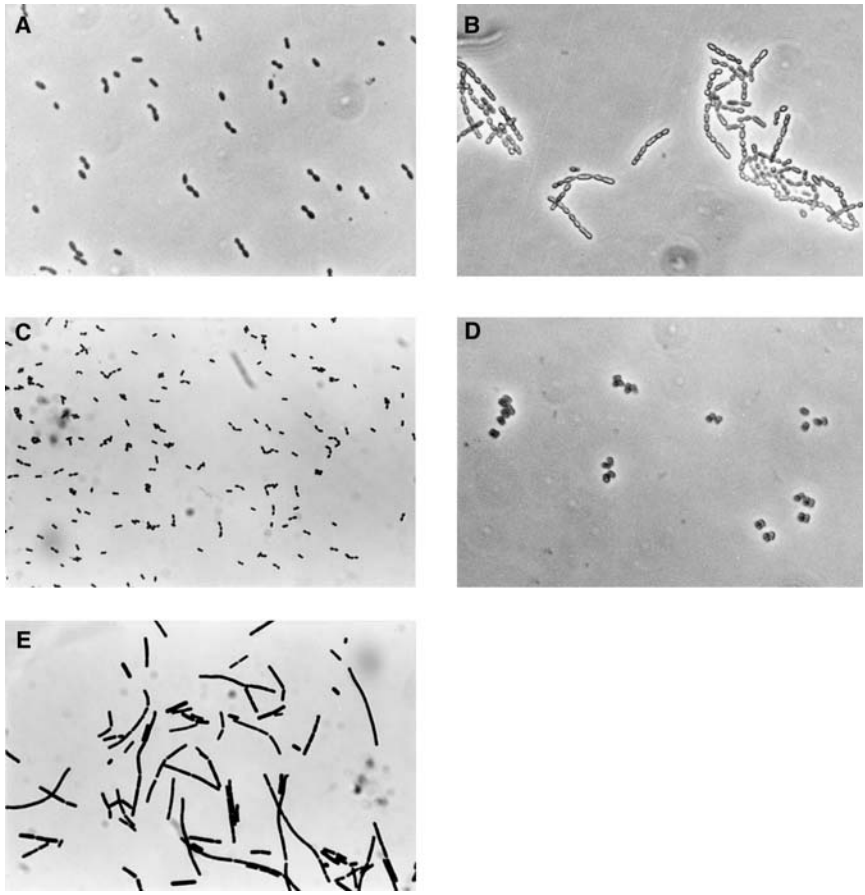


Figure 10.1 Photograph of lactic acid bacteria: (a) *Lactococcus lactis*, (b) *Streptococcus thermophilus*, (c) *Leuconostoc mesenteroides*, (d) *Pediococcus acidilactici*, and (e) *Lactobacillus acidophilus*.

anaerobes and in glucose broth can reduce the pH to 4.0 and produce L(+)-lactic acid. They ferment fructose, mannose, and lactose, but generally not galactose and sucrose. Cells survive 60ºC for 30 min. Their natural habitat is unknown, although they are found in milk.

C. *Leuconostoc*

The Gram-positive cells are spherical to lenticular, arranged in pairs or in chains, nonmotile, nonsporulating, catalase negative, and facultative anaerobes (Figure 10.1). The species grow well between 20 and 30ºC, with a range of 1 to 37ºC. Glucose is fermented to D(–)-lactic acid, CO_2, ethanol, or acetic acid, with the pH reduced to 4.5 to 5.0. The species grow in milk but may not curdle. Also, arginine is not hydrolyzed. Many form dextran while growing on sucrose. Citrate is utilized to produce diacetyl and CO_2. Some species can survive 60ºC for 30 min. *Leuconostoc* species are found in plants, vegetables, silage, milk and some milk products, and raw and processed meats.[2,4]

At present, five species are known: *Leuconostoc mesenteroides*, *Leu. paramesenteroides*, *Leu. lactis*, *Leu. carnosum*, and *Leu. gelidum*. *Leu. mesenteroides* has three subspecies: subsp. *mesenteroides*, ssp. *dextranicum*, and ssp. *cremoris*. *Leu. mesenteroides* ssp. *cremoris* and *Leu. lactis* are used in some dairy and vegetable fermentations. Many of these species, particularly *Leu. carnosum* and *Leu. gelidum*, have been associated with spoilage of refrigerated vacuum-packaged meat products.[5]

Leuconostocs are morphologically heterogenous and may contain genetically diverse groups of bacteria. Recently, two new genera have been created from it: *Weisella* and *Oenococcus*. *Oen. oeni* is found in wine and related habitant and is used for malolactic fermentation in wine.

D. *Pediococcus*

The cells are spherical and form tetrads, but they can be present in pairs. Single cells or chains are absent (Figure 10.1). They are Gram-positive, nonmotile, nonsporulating, facultative anaerobes. They grow well between 25 and 40°C; some species grow at 50°C. They ferment glucose to L(+)- or DL-lactic acid, some species reducing the pH to 3.6. Depending on the species, they can ferment sucrose, arabinose, ribose, and xylose. Lactose is not generally fermented, especially in milk, and milk is not curdled.[2] Some strains may have weak lactose-hydrolyzing capability, especially in broth containing lactose as a carbohydrate source.

Depending on the species, they are found in plants, vegetables, silage, beer, milk, and fermented vegetables, meats, and fish. The genus has seven to eight species, of which *Ped. pentosaceus* and *Ped. acidilactici* are used in vegetables, meat, cereal, and other types of fermented foods. They have also been implicated in ripening and flavor production of some cheeses as secondary cultures. These two species are difficult to differentiate, but compared with *Ped. acidilactici*, *Ped. pentosaceus* ferments maltose, does not grow at 50ºC, and is killed at 70ºC in 5 min.[2] *Ped. halophilus*, used in fermentation of high-salt products, is now classified as *Tet. halophilus*.

E. *Lactobacillus*

The genus *Lactobacillus* includes a heterogenous group of Gram-positive, rod-shaped, usually nonmotile, nonsporulating, facultative anaerobic species that vary widely morphologically and in growth and metabolic characteristics (Figure 10.1). Cells vary from very short (almost coccoid) to very long rods, slender or moderately thick, often bent, and can be present as single cells or in short to long chains. While growing on glucose, depending on a species, they produce either only lactic acid [L(+), D(–), or DL] or a mixture of lactic acid, ethanol, acetic acid, and CO_2. Some also produce diacetyl. Many species utilize lactose, sucrose, fructose, or galactose, and some species can ferment pentoses. Growth temperature can vary from 1 to 50ºC, but most that are used as starter cultures in controlled fermentation of foods grow well from 25 to 40ºC. Several species involved in natural fermentation of some foods at low temperature can grow well from 10 to 25ºC. While growing in a metabolizable carbohydrate, depending on a species, the pH can be reduced between 3.5 and 5.0.

They are distributed widely and can be found in plants; vegetables; grains; seeds; raw and processed milk and milk products; raw, processed, and fermented meat products; and fermented vegetables; some are found in the digestive tract of humans, animals, and birds. Many have been associated with spoilage of foods.

Among the large number of species, some have been used in controlled fermentation (dairy, meat, vegetables, and cereal), some are known to be associated with natural fermentation of foods, a few are consumed live for their beneficial effect on intestinal health, and some others have an undesirable effect on foods. On the basis of their metabolic patterns of hexoses and pentoses (discussed in Chapter 11), the species have been divided into three groups (Table 10.1).[2] Those in Group I ferment hexoses (and disaccharides such as lactose and sucrose) to produce mainly lactic acids and do not ferment pentoses (such as ribose, xylose, or arabinose). Those in

Table 10.1 Division of *Lactobacillus* Species into Groups

Characteristics	Group I	Group II	Group III
Previous designation	Thermobacterium	Streptobacterium	Betabacterium
Carbohydrate fermentation pattern[a]	Obligately homofermentative	Facultatively heterofermentative	Obligately heterofermentative
End products of carbohydrate fermentation	Lactate	Lactate or lactate, acetate, ethanol, CO_2, formate	Lactate, acetate, ethanol, CO_2
Ferment pentoses	–	+	+
Representative species	*Lab. delbrueckii* ssp. *delbrueckii, bulgaricus, lactis; Lab. leichmannii; Lab. acidophilus, Lab. helveticus*	*Lab. casei* ssp. *casei, rhamnosus, pseudoplanatarum; Lab. plantaru; Lab. curvatus; Lab. sake*	*Lab. fermentum; Lab. divergens; Lab. kefir; Lab. confuses; Lab. brevis; Lab. sanfrancisco; Lab. reuteri*

[a] Homofermentatives (produce mainly lactic acid) and heterofermentatives (produce lactic acid as well as large amounts of other products).

Group II, depending on the carbohydrates and the amounts available, either produce mainly lactic acid, or a mixture of lactic, acetic, and formic acids, ethanol, and CO_2. Group III species ferment carbohydrates to a mixture of lactate, acetate, ethanol, and CO_2.

The three *Lactobacillus delbrueckii* subspecies are used in the fermentation of dairy products, such as some cheeses and yogurt. They grow well at 45ºC and ferment lactose to produce large amounts of D(–) lactic acid. β-galactosidase in these subspecies is constitutive. *Lab. acidophilus* and *Lab. reuteri* are considered beneficial intestinal microbes (probiotic) and present in the small intestine. *Lab. acidophilus* is used to produce fermented dairy products and also either added to pasteurized milk or made into tablets and capsules for consumption as probiotics. It metabolizes lactose, and produces large amounts of D(–)-lactic acid. However, in *Lab. acidophilus*, β-galactosidase is generally inducible. *Lab. helveticus* is used to make some cheeses and ferment lactose to lactic acid (DL). *Lab. casei* ssp. *casei* is used in some fermented dairy products. It ferments lactose and produces L(+)-lactic acid. Some strains are also used as probiotic bacteria. Strains of *Lab. casei* ssp. *rhamnosus* (also called *Lab. rhamnosus*) are now used as a probiotic bacterium. Some strains of *Lab. johnsonii* are also used in probiotics. *Lab. plantarum* is used in vegetable and meat fermentation. It produces DL-lactic acid. *Lab. curvatus* and *Lab. sake* can grow at low temperatures (2 to 4ºC) and ferment vegetable and meat products. *Lab. sake* is used to ferment sake wine. *Lab. kefir* is important in the fermentation of kefir, an ethnic fermented sour milk. *Lab. sanfrancisco* is associated with other microorganisms in the fermentation of San Francisco sourdough bread. *Lab. viridescens, Lab. curvatus,* and *Lab sake* are associated with spoilage of refrigerated meat products.[2,4]

F. *Oenococcus*

Oen. oeni, previously designated as *Leu. oeni,* has the general characteristics of *Leuconostoc* spp. It is found in the winery environment. It is sometimes used to accelerate malolactic fermentation in wine. The cells transport malate in wine and metabolize it to lactic acid and CO_2. This process reduces the acidity of wine.

IV. OTHER STARTER CULTURES

A. *Bifidobacterium*

They are morphologically similar to some *Lactobacillus* spp. and were previously included in the genus *Lactobacillus*. The cells are Gram-positive, rods of various shapes and sizes, present as single cells or in chain of different sizes. They are nonsporeforming, nonmotile, and anaerobic, although some can tolerate O_2 in the presence of CO_2. The species grow optimally at 37 to 41ºC, with a growth temperature range of 25 to 45ºC. They usually do not grow at a pH above 8.0 or below 4.5. They ferment glucose to produce lactic and acetic acid in a 2:3 molar ratio without producing CO_2, and also ferment lactose, galactose, and some pentoses.[2]

They have been isolated from feces of humans, animals, and birds and are considered beneficial for the normal health of the digestive tract. They are present in large numbers in the feces of infants within 2 to 3 d after birth, and usually present in high numbers in breast-fed babies. They are usually found in the large intestine.

Many species of this genus have been isolated from the feces of humans and animals. Some of these include *Bifidovacterium bifidum, Bif. longum, Bif. brevis, Bif. infantis,* and *Bif. adolescentis*. All have been isolated in humans; however, some species are more prevalent in infants than in adults. Some of these species have been added to dairy products to supply live cells in high numbers to restore and maintain intestinal health in humans.

B. *Propionibacterium*

The genus includes species in the classical or dairy propionibacterium group and the cutaneous or acne propionibacterium group. Here, only the diary group is discussed.[2]

The cells are Gram-positive, pleomorphic thick rods 1 to 1.5 μm in length, and occur in single cells, pairs, or short chains with different configurations. They are nonmotile, nonsporulating, anaerobic (can also tolerate air), catalase positive, and ferment glucose to produce large amounts of propionic acid and acetic acid. They also, depending on the species, ferment lactose, sucrose, fructose, galactose, and some pentoses. They grow optimally at 30 to 37ºC. Some species form pigments. They have been isolated from raw milk, some types of cheeses, dairy products, and silage.

At present, four species of diary propionibacterium are included in the genus: *Propionibacterium freudenreichii, Pro. jensenii, Pro. thoenii*, and *Pro. acidipropionici*. All four are associated with natural fermentation of Swiss-type cheeses, but *Pro. freudenreichii* has been used as a starter culture in controlled fermentation.

C. *Brevibacterium*

The genus contains a mixture of coryniform bacterial species, some of which have important applications in cheese production and other industrial fermentations. *Brevibacterium linens* is used in cheese ripening as it has extracellular proteases. The cells are nonmotile, Gram-positive, and capable of growing in high salt and wide pH ranges.

D. *Acetobacter*

A species in this genus, *Ace. aceti*, is used to produce acetic acid from alcohol.[2] The cells are Gram-negative; aerobic; rods (0.5 to 1.5 μm); occurring as single cells, pairs, or chains; and can be motile or nonmotile. They are obligate aerobes, catalase positive, and oxidize ethanol to acetic acid and lactic acid to CO_2 and H_2O. They grow well from 25 to 30ºC. They are found naturally in fruits, sake, palm wine, cider, beer, sugar cane juice, tea fungus, and soil. Some species synthesize large amounts of cellulose.[2]

V. YEASTS AND MOLDS

Many yeasts and molds are important in food, but most are involved with the spoilage of food and mycotoxin production (by molds). Several are, however, used in food bioprocessing. At present, genetic improvements are being made to improve their desirable characteristics. A brief discussion is provided here for some that are used in food fermentation.

A. Yeasts

Among the many types of yeasts, only a few have been associated with fermentation of foods and alcohol, production of enzymes for use in food, production of SCPs, and as additives to impart desirable flavor in some foods. The most important genus and species used is *Saccharomyces cerevisiae*.[6,7] It has been used to leaven bread and produce beer, wine, distilled liquors, and industrial alcohol; produce invertase (enzyme); and flavor some foods (soups). However, many strains have been developed to suit specific needs.

The cells are round, oval, or elongated. They multiply by multipolar budding or by conjugation and formation of ascospores. The strains are generally grouped as bottom yeasts or top yeasts. Top yeasts grow very rapidly at 20ºC, producing alcohol and CO_2. They also form clumps that, because of rapid CO_2 production, float at the surface. In contrast, bottom yeasts grow better at 10 to 15ºC, produce CO_2 slowly (also grow slow), do not clump, and thus settle at the bottom. Top yeasts and bottom yeasts are used according to the need of a particular fermentation process.

Candida utilis has been used to produce SCPs. It is a false yeast (*Fungi imperfecti*) and reproduces by budding (not by conjugation). The cells are oval to elongated and form hyphae with large numbers of budding cells. They are also involved in food spoilage.

Kluyveromyces marxianus and *Klu. marxianus* var. *lactis* can hydrolyze lactose and have been associated with natural fermentation, along with other yeasts and lactic acid bacteria, of alcoholic dairy products such as kefir. They have also been associated with spoilage of some dairy products.[6] At present, they are used to produce β-galactosidase (lactase) for use commercially to hydrolyze lactose. The enzyme is now used to produce low-lactose milk.

B. Molds

Although most molds are associated with food spoilage and many form mycotoxins while growing in foods, other species and strains are used in processing of foods and to produce additives and enzymes for use in foods.[7]

In general, molds are multicellular, filamentous fungi. The filaments (hyphae) can be septate or nonseptate and have nuclei. They divide by elongation at the tip of a hypha (vegetative reproduction) or by forming sexual or asexual spores on a spore-bearing body.

Among many genera, several species from genera *Aspergillus* and *Penicillium*, and a few from *Rhizopus* and *Mucor,* have been used for beneficial purposes in food.

Strains to be used for such purposes should not produce mycotoxins. It is difficult to identify a nonmycotoxin-producer strain in the case of natural fermentation, but should be an important consideration in the selection of strains for use in controlled fermentation.

Aspergillus oryzae is used in fermentation of several oriental foods, such as sake, soy sauce, and miso. It is also used as a source of some food enzymes. *Asp. niger* is used to produce citric acid and gluconic acid from sucrose. It is also used as a source of the enzymes pectinase and amylase. *Penicillium roquefortii* is used for ripening of Roquefort, Gorgonzola, and blue cheeses. Some strains can produce the neurotoxin roquefortin. In the selection and development of strains for use in cheese, this aspect needs careful consideration. *Pen. camembertii* is used in Camembert cheese and *Pen. caseicolum* is used in Brie cheese. They are also used to produce the enzyme glucose oxidase.

VI. CONCLUSION

Food-grade bacteria, yeasts, and molds are used in different combinations to produce several thousands of fermented foods worldwide by natural or controlled fermentation of milk, meat, fish, egg, fruits, vegetables and others. The species and strains used as starter cultures in controlled fermentation should not only be safe and regulated but also be able to produce desirable characteristics in the fermented foods. These characteristics are the result of metabolic breakdown of carbohydrates, proteins, and lipids present in the food. Some of these are discussed briefly in Chapter 11 (also see Chapter 7).

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QUESTIONS

1. Discuss the criteria used to select a microorganism for beneficial purposes in foods.
2. List the different ways microorganisms are used beneficially in foods.
3. List the genera that are now included in the group of lactic acid bacteria.
4. List the genera currently used as starter cultures in food fermentation.
5. List one species each from *Lactococcus* and *Streptococcus*, and two each from *Leuconostoc, Pediococcus, Bifidobacterium*, and *Propionibacterium*, used in food fermentation.
6. How are the species in genus *Lactobacillus* (the basis) divided into groups? List two species from each group.
7. When are the following terms used: lactococci and *Lactococcus*; streptococci and *Streptococcus*; leuconostocs and *Leuconostoc*; pediococci and *Pediococcus*; and lactobacilli and *Lactobacillus*?
8. Name three bacterial species that are now used as probiotic bacteria.
9. In yeast fermentation of different foods and beverages, only one species is used. Name the species, and discuss how one species can be effective in so many fermentation processes. Discuss the characteristics of bottom and top yeasts. Name a species that is now used commercially to produce β-galactosidase (lactase).
10. How are molds used in different ways in food? Name two species and list their uses. What precautions are needed while using a mold strain in food fermentation?

CHAPTER 11

Biochemistry of Some Beneficial Traits

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I. INTRODUCTION

The beneficial microorganisms metabolize some of the components present in the starting materials (i.e., food, such as milk or meat) to produce energy and cellular materials and to multiply. In the process, they produce some end products that are no longer necessary for the cells, and therefore these by-products are excreted in the environment. Some of these by-products impart unique characteristics (mostly texture and flavor) to the remaining components of the starting materials. These are

bioprocessed or fermented products and are considered desirable by consumers. Some of the by-products of fermentation can also be purified and used as food additives. The production of several of these by-products by some desirable microorganisms is discussed in this chapter. (See also Chapter 7.)

Before describing the metabolic pathways used by these microbes, it will be helpful to review the components of foods (substrates) used in fermentation. Before the substrates are metabolized inside microbial cells, they have to be transported from the outside environment. It will be beneficial to recognize, in brief, the cellular components involved in the transport of these substrates.

The important substrates available in the starting materials of fermentation include several carbohydrates, proteinaceous and nonprotein nitrogenous (NPN) compounds, and lipids. The important fermentable carbohydrates in foods are starch, glycogen (in meat), lactose (in dairy products), sucrose, maltose (from breakdown of starch), glucose, fructose, and pentoses (from plant sources). The proteinaceous and NPN components include mainly large proteins (both structural and functional), peptides of different sizes, and amino acids. Lipids can include triglycerides, phospholipids, fatty acids, and sterols. Microorganisms differ greatly in their ability to transport these components from outside and metabolize them inside the cells.

II. MECHANISMS OF TRANSPORT OF NUTRIENTS

Nutrient molecules have to pass through the cell barriers — the cell wall and the cell membrane. However, in most Gram-positive lactic acid bacteria, the main barrier is the cytoplasmic membrane. The cytoplasmic membrane is made up of two layers of lipids in which protein molecules are embedded, some of which span the lipid bilayer from the cytoplasmic side to the cell wall side (Figure 11.1 and Figure 2.2). Many of them are transport proteins involved in carrying nutrient molecules from the outside into the cell (also removing many by-products from the cell into the environment).[1,2]

In general, small molecules, such as mono- and disaccharides, amino acids, and small peptides (up to 8 to 10 amino acids), are transported almost unchanged inside the cell by specific transport systems, either singly or in groups. Fatty acids (either free or hydrolyzed from glycerides) can dissolve and diffuse through the lipid bilayers. In contrast, large carbohydrates (polysaccharide such as starch), large peptides, and proteins (such as casein, albumen) cannot be transported directly inside the cell. If a cell is capable of producing specific extracellular hydrolyzing enzymes that are either present on the surface of the cell wall or released into the environment, then large nutrient molecules can be broken down to small molecules and then transported by the appropriate transport systems.

Mono- and disaccharides, amino acids, and small peptides are transported through the membrane by different active transport systems, such as primary transport systems (e.g., ATP-binding cassette or ABC transporters), secondary transport systems (e.g., uniport, symport and antiport systems that use proton motive force), and phosphoenolpyruvate-phosphotransferase (PEP-PTS) systems.[3] A system can be specific for a type of molecule or for a group of similar molecules (group transfer),

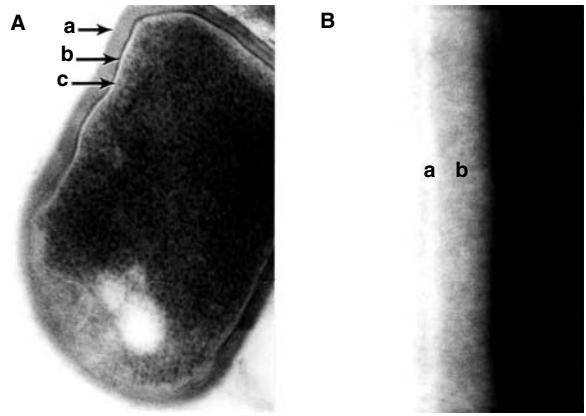


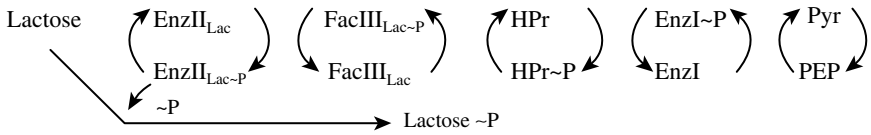
Figure 11.1 A) Transmission electron microscopy photograph of a thin section of *Lactobacillus acidophilus* cell showing the (a) anionic (teichoic, teichouronic, lipoteichoic acids) polysaccharide layer, (b) mucopeptide layer, and (c) cytoplasmic membrane. (B) Negatively stained electron microscopy photograph of *Lactobacillus acidophilus* showing (a) surface layer protein and (b) cell wall. Cytoplasm is stained dark.

can transport against the concentration gradient of a substrate, and the transport process requires energy. In the PEP-PTS system for PTS-sugars, the energy is derived from PEP; in the permease system (for permease sugars, amino acids, and probably small peptides), energy is derived from proton motive force.

III. TRANSPORT AND METABOLISM OF CARBOHYDRATES

In lactic acid bacteria and other bacteria used in food fermentation, disaccharide and monosaccharide (both hexoses and pentoses) molecules can be transported by PEP-PTS as well as by permease systems.[1–6] The same carbohydrate can be transported by the PEP-PTS system in one species and by the permease system in another species. Similarly, in a species, some carbohydrates are transported by the PEP-PTS system, whereas others are transported by the permease system.

A. PEP-PTS System for Lactose Transport in *Lactococcus lactis*



The high-energy phosphate from PEP is transferred sequentially to EnzI, HPr (both in the cytoplasm and nonspecific for lactose), FacIII$_{Lac}$, and EnzII$_{Lac}$ (both on the membrane and specific for lactose), and finally to lactose. Lactose from the environment is transported in the cytoplasm as lactose-phosphate (galactose-6-phosphate-glucose).

B. Permease System for Lactose in *Lactobacillus acidophilus*

$$\begin{array}{c} \text{Lactose}+[H^+] \\ \downarrow \\ \text{Permease}_{\text{Lac}} \rightarrow [\text{Permease:Lactose:}H^+] \rightarrow \text{Lactose} + [H^+] \end{array}$$

One molecule of lactose carries one H^+ with it in the $\text{permease}_{\text{Lac}}$ molecule (specific for lactose). Once inside, there is a conformation change in the permease molecule, which causes release of the lactose molecule and H^+ inside the cytoplasm. Release of lactose and H^+ causes permease to change to original conformation. Lactose is transported as lactose (galactose–glucose).

C. Carbohydrates Available inside the Cells for Metabolism

Mono- and disaccharides are transported from the environment inside the cells either by the permease or PEP-PTS system. In food fermentation, they generally include several pentoses, glucose, fructose, sucrose, maltose, and lactose. Pentoses and hexoses are metabolized by several different pathways, as described later. Sucrose, maltose, and lactose, the three disaccharides, are hydrolyzed inside the cell by the enzymes sucrase, maltase, and lactase (β-galactosidase), respectively, to hexoses. Lactose-P (galactose-6-phosphate-glucose) is hydrolyzed by phospho-β-galactosidase to yield glucose and galactose-6-phosphate before further metabolism.

D. Homolactic Fermentation of Carbohydrates

Hexoses in the cytoplasm, either transported as hexoses or derived from disaccharides, are fermented by homolactic species of lactic acid bacteria to produce mainly lactic acid. Theoretically, one hexose molecule will produce two molecules of lactate. The species include genera from *Lactococcus, Streptococcus, Pediococcus*, and Group I and Group II *Lactobacillus* (Table 11.1; also see Table 10.1). Hexoses are metabolized through Embden–Meyerhoff–Parnas (EMP) pathway (Figure 11.2). These species have fructose diphosphate (FDP) aldolase, which is necessary to hydrolyze a 6C hexose to yield two molecules of 3C compounds. They also lack a key enzyme, phosphoketolase (in HMS pathway), present in species that are heterolactic fermentors.[3–7]

In the EMP pathway, with glucose as the substrate, two ATP molecules are used to convert glucose to fructose-1,6-diphosphate (Figure 11.2). Hydrolysis of these molecules generates two molecules of 3C compounds. Subsequent dehydrogenation (to produce NADH + H^+ from NAD), phosphorylation, and generation of two ATP molecules lead to production of PEP (can be used in PEP-PTS sugar transport). Through the generation of substrate-level ATP, PEP is converted to pyruvate, which, by the action lactate dehydrogenase, is converted to lactic acid. The ability of a lactic acid bacterial species to produce L(+)-, D(−)-, or DL-lactic acid is determined by the type of lactate dehydrogenase (L, D, or a mixture of both, respectively) it contains.

Table 11.1 Fermentation of Monosaccharides by Some Starter-Culture Bacteria to Produce Different By-Products

Genus	Monosaccharide	Fermentation	Pathway[a]	Main Products
Lactococcus	Hexoses	Homolactic	EMP	Lactate
Streptococcus	Hexoses	Homolactic	EMP	Lactate
Pediococcus	Hexoses	Homolactic	EMP	Lactate
Leuconostoc	Hexoses	Heterolactic	HMS	Lactate, CO_2, acetate/ethanol (1:1:1)
	Pentoses	Heterolactic	PP	Lactate, acetate/ethanol (1:1)
Lactobacillus				
Group I	Hexoses	Homolactic	EMP	Lactate
Group II	Hexoses	Homolactic	EMP	Lactate
	Pentoses	Heterolactic	PP	Lactate, acetate/ethanol (1:1)
Group III	Hexoses	Heterolactic	HMS	Lactate, CO_2, acetate/ethanol (1:1:1)
	Pentoses	Heterolactic	PP	Lactate, acetate/ethanol (1:1)
Bifidobacterium	Hexoses	Heterolactic	BP	Lactate, acetate (1:1.5)

[a]EMP, Embden–Meyerhoff–Parnas; HMS, hexose monophosphate shunt (also called phospho-gluconate-phosphoketolase); PP, pentose-phosphate, BP, bifidus (also called fructose ketolase).

The overall reaction involves the production of two molecules each of lactic acid and ATP from one molecule of hexose. The lactic acid is excreted in the environment.

Other hexoses, such as fructose (transported as fructose or from hydrolysis of sucrose), galactose (from hydrolysis of lactose), and galactose-6-phosphate (from hydrolysis of lactose-phosphate following transport of lactose by the PEP-PTS system), undergo different molecular conversion before they can be metabolized in the EMP pathway. Thus, fructose is phosphorylated by ATP to fructose-6-phosphate before being used in the EPM pathway. Galactose is converted first to galactose-1-phosphate, then to glucose-1-phosphate, and finally to glucose-6-phosphate through the Leloir pathway before entering the EMP pathway. Galactose-6-phosphate is first converted to tagatose-6-phosphate, then to tagatose-1, 6-diphosphate, and then hydrolyzed to dihydroacetone phosphate and glyceraldehyde-3-phosphate by the tagatose pathway before entering the EMP pathway.

Besides being an important component in the production of fermented foods (e.g., yogurt, cheeses, and fermented sausage and vegetables), lactic acid is used as an ingredient in many foods (e.g., processed meat products).[7] For this purpose, L(+)-lactic acid is preferred and approved by regulatory agencies as a food additive (as it is also produced by the muscle). Lactic acid bacteria capable of producing L(+)-

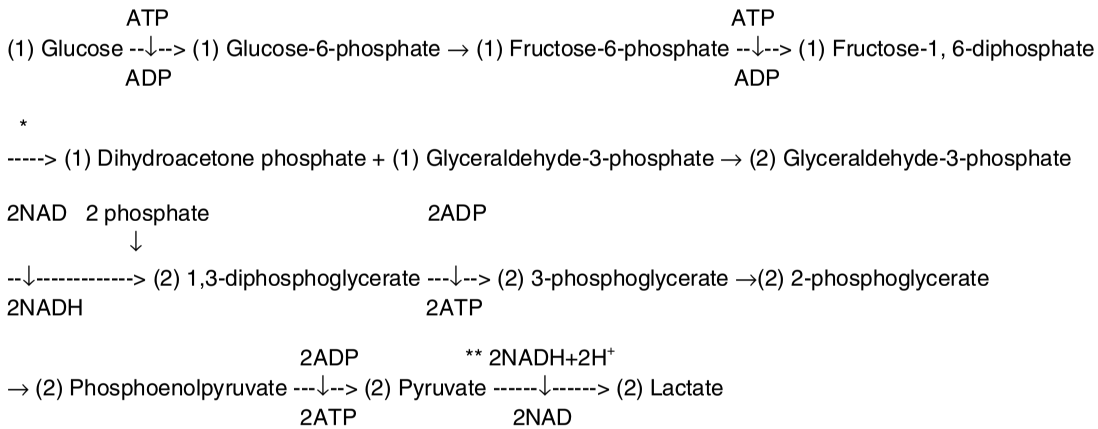


Figure 11.2 Homolactic fermentation of hexoses through the EMP pathway.

lactic acid in large amounts (mainly some *Lactobacillus* spp.) are commercially used for this purpose. At present, genetic studies are being conducted to develop strains by inactivating the D-lactate dehydrogenase system in species that have both the L and D systems and produce large amounts of a mixture of L(+)- and D(–)-lactic acid. Some of the species that produce L(+)-lactic acid (>90% or more) are *Lactococcus lactis* ssp. *lactis* and *cremoris*, *Streptococcus thermophilus*, *Lactobacillus. amylovorus, Lab. amylophilus, Lab. casei* spp. *casei, Lab. casei* spp. *rhamnosus,* and *Lab. divergens.* Some of the common species used in food fermentation, namely *Pediococcus acidilactici, Ped. pentosaceus, Lab. delbrueckii* spp. *bulgaricus* and *helvaticus, Lab. acidophilus, Lab. reuteri,* and *Lab. plantarum,* produce a mixture of D(–)- and L(+)-lactic acids, with 20 to 70% being L(+)-lactic acid.[2]

E. Heterolactic Fermentation of Carbohydrates

Hexoses are metabolized to produce a mixture of lactic acid, CO_2, and acetate or ethanol by heterofermentative lactic acid bacteria (Table 11.1). Species from genera *Leuconostoc* and Group III *Lactobacillus* lack fructose diphosphoaldolase (of EMP pathway), but have glucose phosphate dehydrogenase and xylulose phosphoketolase enzymes, which enable them to metabolize hexoses through phosphogluconate-phosphoketolase pathway (or hexose monophosphate shunt) to generate energy.[3–8]

This pathway has an initial oxidative phase followed by a nonoxidative phase (Figure 11.3). In the oxidative phase, glucose following phosphorylation is oxidized to 6-phosphogluconate by glucose phosphate dehydrogenase and then decarboxylated to produce one CO_2 molecule and a 5C compound, ribulose-5-phosphate. In the nonoxidative phase, the 5C compound is converted to xylulose-5-phosphate, which, through hydrolysis, produces one glyceraldehyde-3-phosphate and one acetyl phosphate. Glyceraldehyde-3-phosphate is subsequently converted to lactate. Acetyl phosphate can be oxidized to yield acetate, or reduced to yield ethanol (depending on the O–R potential of the environment). Species differ in their abilities to produce ethanol, acetate, or a mixture of both. The end products are excreted into the environment.

F. Metabolism of Pentoses

The species in genera *Leuconostoc* and Group III *Lactobacillus* can ferment different pentose sugars by the pentose-phosphate pathway to produce ATP, lactate, and acetate, because they have the phosphoketolase enzyme. In Group II *Lactobacillus,* this enzyme is inducible and is produced only when a pentose is present in the environment. Although *Ped. pentosaceus, Ped. acidilactici*, and *Lac. lactis* can metabolize some pentoses, the pathways are not clearly known.[4–6,8]

The metabolizable pentoses by the *Leuconostoc* and *Lactobacillus* (Group II and Group III) are first converted to xylulose-5-phosphate by several different ways. Xylulose-5-phosphate is then metabolized to produce lactate and acetate or ethanol by the mechanisms described in the nonoxidizing portion of metabolism of hexoses by heterofermentative lactic acid bacteria (Figure 11.3). No CO_2 is produced from the metabolism of pentoses through this pathway.

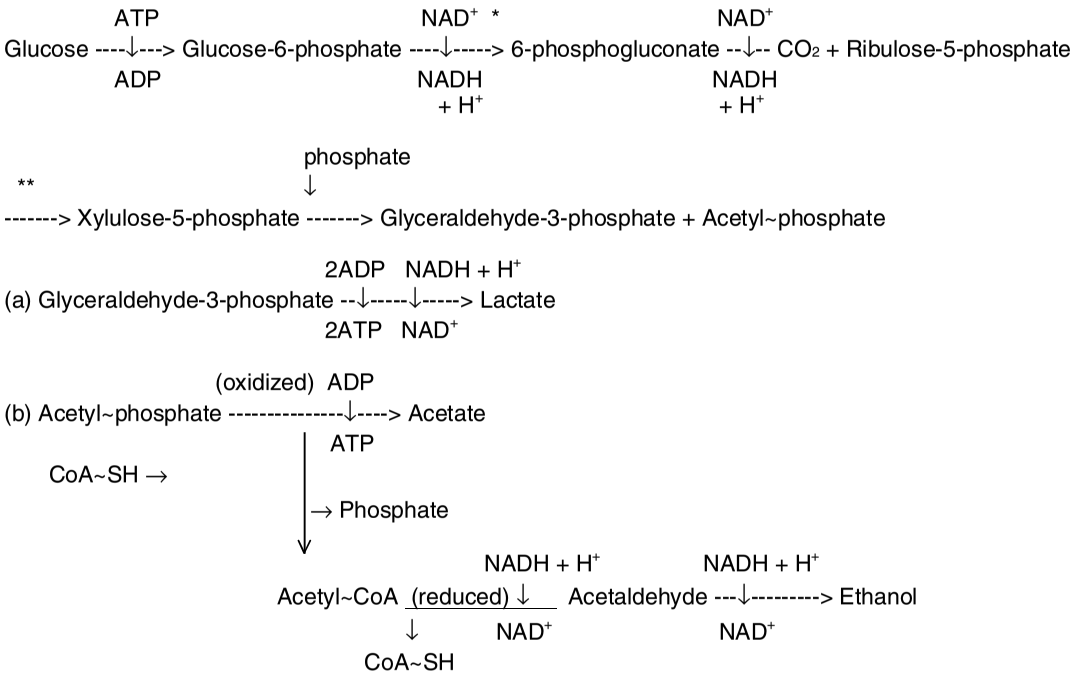


Figure 11.3 Heterolactic fermentation of hexoses through HMS.

G. Hexose Fermentation by *Bifidobacterium*

Bifidobacterium species metabolize hexoses to produce lactate and acetate by the fructose–phosphate shunt or bifidus pathway.[1–3] For every two molecules of hexoses, two molecules of lactate and three molecules of acetate are produced without generation of any CO_2 (Figure 11.4). From two molecules of fructose-6-phosphate, generated from two molecules of glucose, one molecule is converted to produce one 4C erythrose-4-phosphate and one acetyl-phosphate (which is then converted to acetate). Another molecule of fructose-6-phosphate combines with erythrose-4-phosphate to generate two molecules of the 5C xylulose-5-phosphate through several intermediate steps. Xylulose-5-phosphates are then metabolized to produce lactates and acetates by the method described in the nonoxidizing part of heterolactic fermentation (also in pentose fermentation; Figure 11.3).

H. Diacetyl Production from Citrate

Diacetyl, a 4C compound, is important in many fermented dairy products for its pleasing aroma or flavor (butter flavor). It is also used separately in many foods to impart a butter flavor. Many lactic acid bacteria can produce it in small amounts from pyruvate, which is generated from carbohydrate metabolism (Chapter 7).[8] However, *Lac. lactis* ssp. *lactis* biovar diacetylactis and *Leuconostoc* species can produce large amounts of diacetyl from citrate (Figure 11.5).[1,3] Citrate, a 6C compound, is transported from outside into the cells by the citrate-permease system. It is then metabolized through pyruvate to acetaldehyde-TPP (thiamin pyrophosphate). It then combines with pyruvate to form α-acetolactate, which is converted to diacetyl with the generation of CO_2. Under a reduced condition, diacetyl can be converted to acetoin, with a loss of desirable flavor. A producer strain can also be genetically modified to produce excess diacetyl (metabolic engineering).[9]

I. Propionic Acid Production by *Propionibacterium*

The desirable flavor of some cheeses (such as Swiss) in which dairy *Propionibacterium* is used as one of the starter cultures is from propionic acid.[1,10] *Propionibacterium* generates pyruvate from hexoses through the EMP pathway, and the pyruvate is used to generate propionic acid (Figure 11.6). Pyruvate and methylmalonyl ~ CoA produce propionyl ~ CoA and oxalacetate. Propionyl ~ CoA is then converted to propionate. Oxalacetate is recycled to generate methylmalonyl ~ CoA through succinyl ~ CoA. *Propionibacterium* also generates acetate and CO_2 (CO_2 contributes to eye formation in Swiss cheese) from pyruvate.

IV. TRANSPORT AND METABOLISM OF PROTEINACEOUS COMPOUNDS AND AMINO ACIDS

Many lactic acid bacteria used in food fermentation have active transport systems for transporting amino acids and small peptides (ca. 8 to 10 amino acids long).[11,12] Large

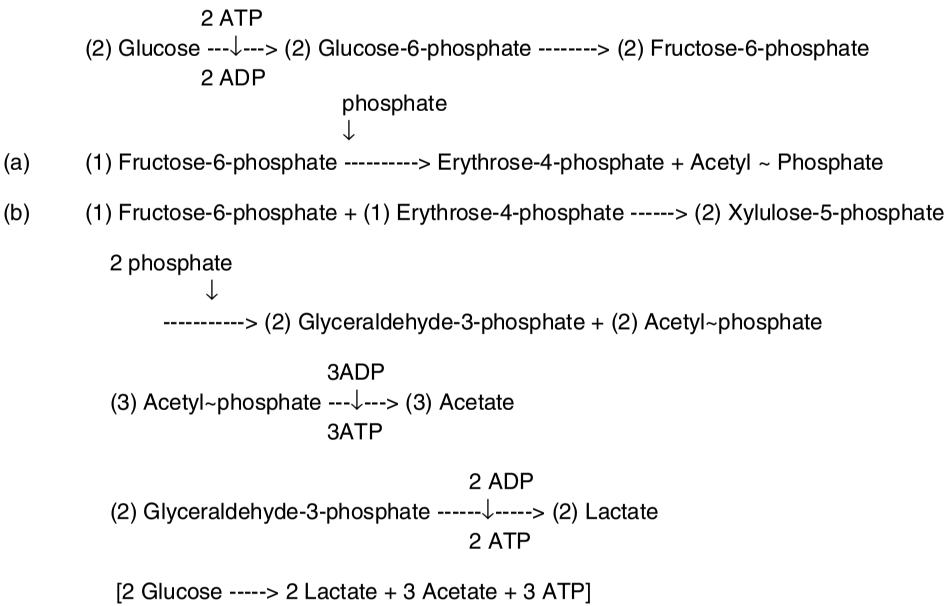

2 ATP
(2) Glucose ---↓---> (2) Glucose-6-phosphate --------> (2) Fructose-6-phosphate
2 ADP
phosphate
↓
(a) (1) Fructose-6-phosphate ----------> Erythrose-4-phosphate + Acetyl ~ Phosphate
(b) (1) Fructose-6-phosphate + (1) Erythrose-4-phosphate ------> (2) Xylulose-5-phosphate
2 phosphate
↓
----------> (2) Glyceraldehyde-3-phosphate + (2) Acetyl~phosphate
3ADP
(3) Acetyl~phosphate ---↓---> (3) Acetate
3ATP
2 ADP
(2) Glyceraldehyde-3-phosphate ------↓-----> (2) Lactate
2 ATP
[2 Glucose -----> 2 Lactate + 3 Acetate + 3 ATP]

Figure 11.4 Hexose fermentation by *Bifidobacterium*.

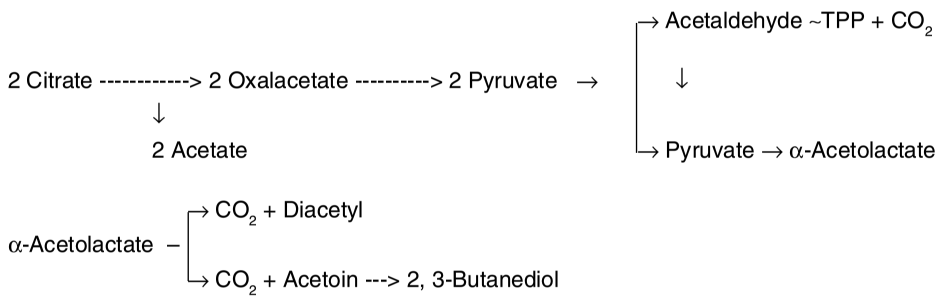


Figure 11.5 Diacetyl production from citrate.

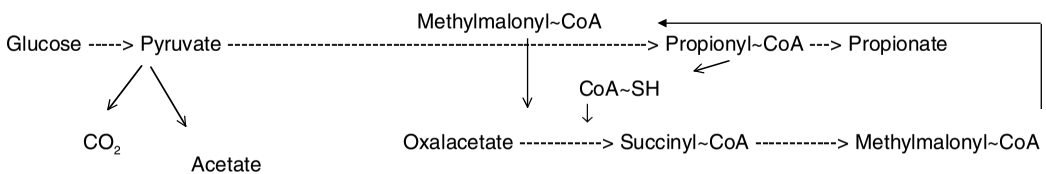


Figure 11.6 Propionic acid metabolism in *Propionibacterium*.

peptides and metabolizable proteins in the environment are hydrolyzed by the proteinase and peptidase enzymes, located mainly on the cell wall of these species, to small peptides and amino acids and then transported, many by active group transport systems, inside the cells. Inside the cells, peptides are converted to amino acids, and the amino acids are then metabolized differently to produce many different end products, which are excreted in the environment. Some of the metabolic processes involve decarboxylation (generates amines, some of which are biologically active, such as histamine from histidine), deamination, oxidative reduction, and anaerobic reduction. Amino acid metabolism by lactic acid bacteria and different associative bacteria during fermentation can produce diverse products, many of which have specific flavor characteristics. Some of these include ammonia, hydrogen sulfide, amines, mercaptans, and disulfides. Many of them, in low concentrations, contribute to the desirable flavor of different fermented foods. Proper hydrolysis of food proteins by the proteolytic enzymes of starter microorganisms is important for the desirable texture of many fermented foods (such as in cheeses). Rapid hydrolysis of proteins can result in production and accumulation of some specific hydrophobic peptides that impart a bitter taste to the product (such as in some sharp Cheddar cheeses).

V. TRANSPORT AND METABOLISM OF LIPID COMPOUNDS

Many starter-culture bacteria metabolize lipids poorly. However, molds have better lipid metabolism systems. Triglycerides and phospholipids are hydrolyzed outside of the cells by lipases, produced by the microorganisms, releasing fatty acids and glycerol and glycerides (mono- or diglycerides). Fatty acids can diffuse through the membrane into the cells and be metabolized. Some fatty acids are incorporated in the membrane. Hydrolysis of glycerides, especially those with small fatty acids such as butyric acid, can cause hydrolytic rancidity of the products. Oxidation of unsaturated fatty acids by microorganisms, especially molds, can produce many flavor compounds, either desirable or undesirable.

VI. CONCLUSION

Many safe and food-grade microbial species are used to produce fermented foods and food additives. They metabolize the carbohydrates, proteins, and lipids in the food by specific pathways and produce metabolites that in turn bring about the desirable acceptance characteristics of the fermented foods and food additives. The metabolic pathways are regulated by many enzymes, the genetic information of which is coded in the genes located in the chromosome and plasmid in a cell. The nature of genes and the mechanisms by which the genetic codes are translated into enzymes in lactic acid bacteria are discussed in Chapter 12.

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QUESTIONS

1. Name two species of yeasts and briefly discuss the use of each in the production of fermented foods and food additives.
2. List three mold species and indicate their specific uses in food bioprocessing.
3. List the main carbohydrates, proteinaceous and NPN compounds, and lipids in food systems that starter-culture bacteria have at their disposal to metabolize.
4. Discuss how lactic acid bacteria, while growing in milk, are able to transport lactose.
5. What are the different types of food carbohydrates transported inside the lactic acid bacterial cells? Explain the functions of β-galactosidase and phospho-β-galactosidase.
6. Define homolactic and heterolactic fermentation of carbohydrates by lactic acid bacteria. Give three examples of lactic acid bacteria in each fermentation group.
7. List the end products (including energy generation) of homolactic and heterolactic fermentation when glucose is used as a substrate. Which enzymes play crucial roles in each pathway?

8. How are galactose, galactose-6-phosphate, and fructose metabolized by EMP pathway by homolactic fermentors? List three species from three genera that produce only L(+)-lactic acid from glucose.
9. Why is bifidus pathway of metabolism of hexose also called fructose–phosphate shunt? What are the end products?
10. How is citrate transported and metabolized to produce diacetyl by some lactic acid bacteria? List two species from two genera that produce diacetyl from citrate. How can other lactic acid bacteria produce diacetyl from carbohydrate metabolism?
11. Pyruvate can be used to produce products other than lactic acid. List four such products and show the reactions involved in their production.
12. Briefly discuss how food proteins and food lipids are transported and metabolized by starter-culture bacteria.

CHAPTER 12

Genetics of Some Beneficial Traits

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I. INTRODUCTION

Since the 1930s, it has been recognized that many important characteristics (traits or phenotypes) in dairy starter-culture bacteria are unstable. For example, a *Lactococcus lactis* strain, while growing in milk, once able to ferment lactose and coagulate the milk, was found to no longer ferment lactose and became useless commercially. Similar losses of other commercially important traits of starter cultures used in dairy and nondairy fermentations, such as ability to hydrolyze proteins (necessary for some cheese production), ability to utilize citrate (for diacetyl production), resistance to bacteriophages, and hydrolysis of sucrose, were observed. However, the specific mechanisms involved in the instability of these important phenotypes were not understood. In the 1960s, the genetic basis of instability of different microbial phenotypes started unfolding. Similar studies, when extended to dairy starter-culture bacteria, revealed the genetic basis of the instability of the important traits. In those days, only a few laboratories were conducting research in the genetics of lactic acid bacteria (notably, Dr. Larry McKay's laboratory at the University of Minnesota). Since the late 1970s, many other laboratories started working in this area and, at present, genetic research of starter-culture bacteria has generated a major interest in many laboratories worldwide. The genetic basis of some of the commercially important phenotypes in some lactic acid bacteria; methods of transfer of desirable traits from one bacterial strain to another to develop a better strain for use in food fermentation; and current advances in genetic studies, such as metabolic engineering and genome sequencing in lactic acid bacteria, are discussed briefly in this chapter.

II. PLASMIDS AND PLASMID-LINKED TRAITS IN STARTER-CULTURE BACTERIA

Starter-culture bacteria, like other bacteria, carry genetic information (genetic code) in the circular chromosomal DNA, circular plasmids, and transposons. Chromosomal DNA carries genetic codes for vital functions of a cell (such as a key enzyme in the EMP or HMS pathway in lactic acid bacteria). Although both plasmid DNA and transposons can carry genetic codes, they are only for nonvital functions, i.e., characteristics that are not absolutely necessary for the survival of a cell (such as the ability to hydrolyze a large protein). However, having such a genetic code gives a strain a competitive advantage over other strains that lack it but share the same environment. Initial research in the early 1970s revealed that many industrially important phenotypes in different lactic acid bacteria are plasmid linked. Since then, because specific techniques are available, the genetic basis of many plasmid-encoded important phenotypes in many starter-culture bacteria, particularly in *Lactococcus lactis*, some *Leuconostoc* spp., and some *Lactobacillus* spp. has been studied. These studies have not only helped identify the locations of many genes, their structure, and the control systems involved in their expression, but also enabled researchers to transfer genes into a cell lacking a specific phenotype and to create a new desirable strain. Characteristics of plasmids and some plasmid-linked traits in starter-culture bacteria are discussed here.[1,2]

A. Important Characteristics of Bacterial Plasmids

- Plasmids are double-stranded, circular, self-replicating DNA that can vary in size (<1 to >100 kb).
- Plasmids may not be present in all species or all strains in a species.
- A strain can have more than one type of plasmid that differ in size and the genetic code it carries.
- A plasmid can be present in more than one copy in a cell (copy number; this is in contrast to a single copy of chromosome that a cell can carry).
- For some plasmids, copy numbers can be reduced (depressed) or increased (amplified) by manipulating the control systems.
- Plasmids can differ in their stability in a cell. A plasmid from a cell can be lost spontaneously or by manipulation.
- Two types of plasmids in a cell may be incompatible, resulting in the loss of one.
- Plasmids can be transferred from one cell (donor) to another compatible cell (recipient) spontaneously or through manipulation.
- Plasmid transfer can occur either only between closely related strains (narrow host range), or between widely related strains from different species or genera (broad host range).
- A plasmid can be cryptic (i.e., not known to carry the genetic code for a known trait).
- Effective techniques for the isolation, purification, molecular weight determination, and nucleotide sequence determination of bacterial plasmids have been developed.
- Genetic codes from different sources (different procaryotes and eucaryotes) can be introduced into a plasmid, which then can be transferred into the cell of an unrelated bacterial species in which the phenotype may be expressed.

B. Some Characteristics of Small (ca. 10 kb) and Large (over 10 to ca. 150 kb) Plasmids

- *Copy Number.* Small plasmids generally occur in multiple copies (10 to 40 per cell); large plasmids are generally present in lesser copies (for a very large plasmid even one copy per cell).
- *Amplification*. Many small plasmids can be amplified to a very high copy number; large plasmids, especially very large ones, cannot be amplified.
- *Conjugal Transfer.* Small plasmids are nonconjugative (but can be transferred along with a conjugative plasmid); large plasmids are generally conjugative.
- *Stability.* Small plasmids are usually unstable; large plasmids are usually stable.
- *Genetic Code.* A large plasmid can encode many phenotypes; a small plasmid can encode only one or a few phenotypes. Plasmids can also have the operon system.

C. Presence of Plasmids in Some Starter-Culture Bacteria

- *Lactococcus Species*. Many strains from both subspecies and the biovar have been analyzed. Most strains have 2 to 10 or more types of plasmids, both small and large.
- *Streptococcus thermophilus*. Among the strains examined, a few strains carry plasmids, generally only one to three types, mostly small and not very large.
- *Leuconostoc Species.* Many species and strains carry plasmids of different sizes, ranging from 1 to 10 or more types.
- *Pediococcus Species*. Limited studies show that the strains either have none or have two to three plasmids, both small and large types.
- *Lactobacillus Species*. Only a limited number of species and strains have been tested. Some species rarely carry plasmids, such as *Lab. acidophilus*. Some carry usually a few, such as *Lab. casei*. Some carry a large number (two to seven) of various sizes, such as *Lab. plantarum*.
- *Bifidobacterium Species.* Limited studies reveal that the species harbor two to five different types of plasmids.
- *Propionibacterium Species.* Limited studies show that some species contain only a few plasmids.

D. Phenotype Assignment to a Plasmid

Following the understanding of plasmid characteristics and the revelation that many starter-culture bacteria carry plasmids, studies were conducted to determine whether a particular plasmid in a strain carries genetic codes necessary for the expression of a specific phenotype or to determine whether a particular phenotype is linked to a specific plasmid in a strain. The loss of lactose-fermenting ability (Lac^+ phenotype) among *Lac. lactis* strains was suspected to be due to loss of a plasmid that encodes genes necessary for lactose hydrolysis (also for lactose transport). The possible linkage of the Lac^+ phenotype to a plasmid in a *Lac. lactis* strain was first studied according to the following protocol:

1. A Lac^+ strain can become Lac^- (inability to hydrolyze lactose) spontaneously or when grown in the presence of a chemical curing agent (e.g., acriflavin) or a physical curing agent (e.g., high temperature).

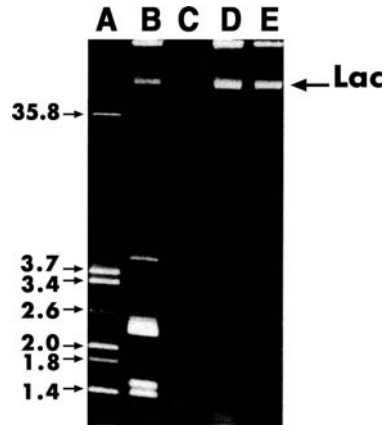


Figure 12.1 Plasmid profiles of *Lactococcus lactis* ssp. *lactis*. Lane A, plasmid standards of different molecular weights (Mda); lane B, donor strain with lac-plasmid (35.5 MDa or 53 kb; Lac^+ Sm^s); Lane C: plasmidless recipient strain (Lac^- Sm^r); Lanes D and E: transconjugants showing lac-plasmid (Lac^+ Sm^r).

2. Analysis of the plasmid profile, i.e., types of plasmids present as determined from their molecular weight (kb), showed that a 53-kb plasmid present in the Lac^+ wild strain was missing in Lac^- cured variant. Thus, loss of this plasmid was correlated with the loss of Lac^+ phenotype in this strain.
3. To determine further that the 53-kb plasmid actually encoded the Lac^+ phenotype, the wild Lac^+ strain was conjugally mated with a plasmidless Lac^- strain, and several Lac^+ transconjugants were obtained. (This aspect is discussed later in mechanisms of DNA transfer.) When these transconjugants were analyzed, all were found to contain the 53-kb plasmid (Figure 12.1). Following curing, the transconjugants were converted to Lac^-, and an analysis showed that these variants no longer had the 53-kb plasmid.
4. From this series of experiments, it was determined that the 53-kb plasmid in the specific *Lac. lactis* strain used encodes Lac^+ phenotype.

Similar studies were conducted to determine plasmid linkage of several other traits in starter-culture bacteria.

E. Plasmid-Linked Traits in Starter-Culture Bacteria

Many starter-culture bacteria, especially *Lac. lactis* strains, have been examined for the plasmid linkage of different phenotypes. The studies have revealed that in these bacteria, many commercially important traits are plasmid-encoded. The following are some traits:

- *Lac. lactis*
  Lac^+, lactose hydrolysis (also lactose transport trait)
  Pro^+, proteinase activity
  Cit^+, citrate hydrolysis (also citrate transport trait)

Bac$^+$, production of several bacteriocins (also their respective immunity, processing, and translocation traits; bacteriocin, like nisin, is encoded in a transposon)
Phage^r, resistance to specific bacteriophages
R/M system, restriction/modification
Resistance to several antibiotics (such as Km^r, resistance to kanamycin)
Metabolism of several carbohydrates (such as Gal$^+$, galactose utilization)
Muc$^+$, mucin production

- *Str. thermophilus*. Plasmid linkage of a phenotype not conclusively known
- *Leuconostoc* Species. Bac$^+$, production of different bacteriocins (also immunity to them)
- *Pediococcus* Species.
  Suc$^+$, sucrose hydrolysis
  Bac$^+$, production of different bacteriocins (also immunity to them and translocation)
- *Lactobacillus* Species.
  Lac$^+$, lactose hydrolysis
  Mal$^+$, maltose hydrolysis
  Bac$^+$, production of some bacteriocins (also immunity to them)
  Muc$^+$, ability to produce mucin
  Resistance to some antibiotics (such as erythromycin, Em^r)
  R/M system, restriction/modification

The same phenotype in a species can be encoded in different-size plasmids.

F. Cryptic Plasmids

Many plasmids of lactic acid bacteria carry genes that encode for specific phenotypes. An example is the plasmid pSMB74 in *Pediococcus acidilactici* H, which encodes genes associated with the production of a bacteriocin, pediocin AcH. However, there are other plasmids in lactic acid bacteria for which no specific phenotypes can be assigned, and they are designated as cryptic plasmids. Some of them are able to integrate in the host chromosome and others are able to replicate in different homologous and heterologous hosts. For this reason, some of them have been used to construct vectors for cloning and expression of genes in lactic acid bacteria and some Gram-positive and Gram-negative bacteria. Plasmids pWVO1 and pSH71 have been extensively used to develop a series of cloning and expression vectors.[3]

G. Plasmid Replication

Plasmids of lactic acid bacteria have a high degree of sequence homology in the origin of replication (*rep A*) and the gene encoding the replication protein (*rep B*). Depending on the size, a plasmid replicates either by a sigma-type or by a theta-type mechanism. In the sigma (or rolling circle) replication mode, replication is initiated by the binding of the plasmid encoding the Rep protein to the origin (plus-origin site). This is followed by a nick in one and fixation of Rep at the 5′ end of the nicked strand. The other strand is replicated from the nick site, causing displacement of the nicked strand, thus forming the shape of a sigma. The single strand is

then ligated, forming a single-stranded circle and a duplex plasmid. The replication of the single strand is then followed to produce a double-stranded plasmid. This process generally occurs in small and cryptic plasmids (ca. 12 kb or less), which are relatively unstable.

In the theta-type replication, initiation starts with the formation of replication forks (as in the chromosome) due to separation of the base pairs. Both strands then simultaneously replicate, either in the same or in opposite directions, and two copies of the plasmids are formed at the end of replication. The plasmids are more than 12 kb, stable with limited host range, and encode for many economically important phenotypes.[3,4]

H. Plasmid Mapping and Sequencing

Initially, the cells of a strain are lysed and plasmids are isolated, purified, and separated on the basis of molecular weight by agarose gel electrophoresis. The plasmid of interest is purified from the gel and used for mapping and sequencing. The purified plasmid is subjected to single and double digestions with a set of restriction endonuclease (RE) enzymes, and the number and molecular weight of the fragments generated after each digestion are determined. These results are used to construct the RE map of the plasmid. Individual fragments are then sequenced to construct the complete nucleotide sequence of the plasmid and to locate the open reading frames (ORFs) and regulatory regions in the plasmid. In many studies, the complete nucleotide sequence of a plasmid, especially if it is large, is not determined. To locate the genes expressing a phenotype, different fragments are cloned in a suitable vector, and after transformation host cells are examined for the expression of the phenotype. The fragment that expresses the phenotype is then sequenced to determine the locations of the genes and the regulatory mechanisms. This process has been simplified in recent years by first purifying the protein coded by a gene and sequencing five or more N-terminal amino acids. From this information, nucleotide probes are constructed, and by PCR techniques the location of the genes in the plasmid is determined; this helps sequence the gene by the several procedures available.

III. GENE TRANSFER METHODS IN STARTER-CULTURE BACTERIA

Once the genetic basis of a phenotype in bacteria was understood, studies were conducted to develop means to transfer the genetic materials from one bacterial cell to another. It is recognized that exchange of genetic materials occurs among bacteria naturally, but at a slower pace. However, if a process of introducing genetic materials can be developed under laboratory conditions, the process of genetic exchange can not only be expedited but also help develop desirable strains. In starter-culture bacteria this will help to develop a strain for a specific fermentation process that carries many desirable phenotypes and the least number of undesirable phenotypes.[5,6]

Results of the studies conducted since the 1970s, initially in *Lac. lactis* subspecies and later in other lactic acid bacteria, revealed that genetic materials can be introduced into bacterial cells by several different mechanisms, some of which are discussed here.

A. Transduction

In this process, a transducing bacteriophage mediates the DNA exchange from one bacterial cell (donor) to another cell (recipient). DNA of some phages (designated as temperate bacteriophages) following infection of a cell can integrate with bacterial DNA and remain dormant (see Chapter 13). When induced, the phage DNA separates out from the bacterial DNA and, on some occasions, can also carry a portion of the bacterial DNA encoding genes in it. When the phage-carrying portion of a bacterial DNA infects a bacterial cell and integrates its DNA with bacterial DNA, the phenotype of that gene is expressed by the recipient cell. Initially, the Lac^+ phenotype from a lactose-hydrolyzing *Lac. lactis* strain carrying a temperate phage was transduced to a Lac^- *Lac. lactis* strain to obtain a Lac^+ transductant. This method has been successfully used to transduce Lac^+ phenotype and several other phenotypes (such as Pro^+) in different strains of *Lac. lactis* subspecies. Investigations show that both chromosomal- and plasmid-encoded genes from bacteria can be transduced. Transduction has been conducted successfully in some strains of *Str. thermophilus* and in strains of several *Lactobacillus* species.

The transduction process in starter-culture bacteria is important to determine location of a gene on the DNA for genetic mapping and to study its characteristics. A temperate phage can be induced spontaneously, resulting in lysis of bacterial cells; thus, it is not very useful in commercial fermentation. Also, this method cannot be applied in species that do not have bacteriophages, such as some *Pediococcus* species.

B. Conjugation

In this process, a donor bacterial cell transfers a replica of a portion of its DNA to a recipient cell. The two cells have to be in physical contact to effect this transfer. If the transferred DNA encodes a phenotype, the transconjugant will have the phenotype. To make DNA transfer possible, the donor cells should have several other genes, such as clumping factor (for a physical contact through clumping) and a mobilizing factor (to enable the DNA to move from a donor to a recipient). The process consists of selecting the right donor and recipient strains, mixing the two cell types in a donor:recipient ratio of 2:1 to 10:1 in several different ways for DNA transfer to occur, and then identify the transconjugants by appropriate selection techniques.

This technique has been used successfully to transfer several plasmid-linked phenotypes in some lactic acid bacteria. The plasmid-linked Lac^+ phenotype was transferred conjugally between two *Lac. lactis* species. The transconjugant was Lac^+ and had the specific plasmid, the loss of which resulted in its phenotype becoming Lac^-. Subsequently, the Lac^+ phenotype located in different plasmids in many *Lac. lactis* subspecies and strains were conjugally transferred to Lac^- strains of the same species. Conjugal transfer of different plasmid-linked traits has also been reported in other lactic acid bacteria, such as the diacetyl production trait in *Lac. lactis* ssp. *lactis* biovar diacetilactis.

The method has several limitations, some of which have been listed previously with the characteristics of plasmids. They include plasmid size, plasmid incompatibility and instability in recipient strains, inability to express in hosts, inability to have proper donors and recipients, and, in some cases, inability to recognize the transconjugant. However, by using a broad-host-range plasmid (e.g., pAMβ1, a plasmid of *Enterococcus* spp. encoding an antibiotic gene), it was shown that plasmid transfer by conjugation is possible among lactic acid bacteria between the same species, between two different species in the same genus, or even between two different species from different genera.

C. Transformation

The method involves extraction and purification of DNA from a donor bacterial strain and mixing the purified DNA with the recipient cells. Some DNA fragments encoding a phenotype are expected to pass through the cell barriers (wall and membrane) and become part of the host DNA expressing the new phenotype. In some Gram-positive bacteria (e.g., *Bacillus* spp.), this method has been effective to transfer certain traits. In lactic acid bacteria, limited studies revealed that the technique was not very effective; however, a modified method was effective. First, *Lac. lactis* cells were treated with lysozyme or mutanolysin, or both, to remove the cell wall and to form protoplasts in a high osmotic medium. The protoplasts were then exposed to purified DNA (chromosomal, plasmid, or phage DNA) in the presence of polyethylene glycol. The growth conditions were then changed for the protoplasts to regenerate the cell wall. The transformants were then detected in a selective medium. By this method, Lac^+ phenotype and Em^r (erythromycin-resistance phenotype) and phage DNA (transfection) were transferred to recipient strains of *Lac. lactis* subspecies. Because of limitations in the success rate, this method is not widely studied in lactic acid bacteria.

D. Protoplast Fusion

The technique involves preparation of protoplasts of cells from two different strains and allowing them to fuse together in a suitable high-osmotic environment. Fusion of cells of the two strains and recombination of the genetic materials may occur. By allowing the protoplasts to regenerate the cell wall and by using proper selection techniques, recombinants carrying genetic information from both strains can be obtained. The technique has been used successfully to produce recombinants of *Lac. lactis* subspecies for both Lac^+ and Em^+ phenotypes. However, because of the low success rate, this technique is not used much in lactic acid bacteria.

E. Electrotransformation

In this method, a suspension of recipient cells in high population levels (10^8 cells/200 µl) is mixed with purified DNA (1 to 2 µg) from a donor strain and then exposed to a high-voltage electric field for a few microseconds. This results in temporary formation of small holes in the cell barrier (membrane) through which purified DNA

can pass. Subsequently, the cells are allowed to repair their damage and express the new phenotype to enable their isolation.

This method has been widely used in many lactic acid bacteria to introduce plasmids from different strains of the same species as well as from separate species and genera. In addition, vectors carrying cloned genes from diverse sources have been successfully introduced in several species of lactic acid bacteria. This is currently the most preferred method to transfer DNA from a source into recipient cells of lactic acid bacteria.

F. Conjugative Transposons

Transposons or transposable elements are segments of DNA in chromosomes or plasmids of bacteria that can move from one site to another. Because of this, they can cause rearrangement in the sequence of chromosomal and plasmid DNA and loss or gain of phenotypes in the hosts. The simplest transposons are known as insertion sequences (IS). Each IS element is an autonomous unit, containing a coding region that has the gene encoding the transposase enzyme necessary for its transposition on the host DNA and an inverted nucleotide sequence repeated at each end. Transposition of an IS element occurs at target sites in host DNA, and after transposition the host DNA contains short direct repeats on either end of an IS element. Larger transposons (Tn) contain different genes, such as those that encode for an antibiotic-resistance phenotype, in the central region and an IS element on each end. Transposons can be conjugative and can be transferred from a donor to a host by the same method used for a conjugative plasmid. When such a transfer occurs, the phenotypes encoded by the resident genes (or genes cloned into it) is also transferred and expressed in a recipient strain.[3]

A few IS elements and Tn have been identified in some strains of lactic acid bacteria. *Lactobacillus casei* S1 contains the insertion element IS1, which has two ORFs and an inverted repeat at either end. It is chromosomally located, and, when inserted in a prophage, the lysogenic phage becomes a virulent phage. The nisin-producing *Lac. lactis* ssp. *lactis* ATCC 11454 contains a conjugative transposon, Tn 5276, in the chromosome, which is ca. 70 kb and contains in the central region the genes necessary for nisin A production (*nis* operon) and sucrose utilization.[7] In addition, some conjugative transposons from several *Streptococcus* and *Enterococcus* species that encode antibiotic-resistance markers, such as Tn 916 (encodes tetracycline resistance), have been transferred to different strains of lactic acid bacteria. They have also been used in genetic recombination studies in lactic acid bacteria.

IV. GENE CLONING

To transfer and express a gene from a donor into a recipient, the usual procedure is to clone the gene into a suitable plasmid or cloning vector and introduce the vector into the recipient cells. In the simplest form in this technique, a DNA segment, carrying the genes, is obtained by digesting the purified DNA of the donor with the

suitable DNA restriction endonucleases and purifying it from the mixture. A suitable plasmid (cloning vector) is selected that has one or several gene markers (such as resistance to an antibiotic or metabolizing a carbohydrate) and a site that can be hydrolyzed, preferably with the same restriction enzymes. The plasmid is digested with this enzyme, mixed with the donor DNA fragment, and incubated for the fragment to align in the opening of the plasmid DNA (Figure 12.2). The open ends are then sealed by using suitable enzymes and nucleotide bases. This plasmid carrying the genes from the donor can then be introduced in a bacterial cell in several ways, most effectively by electroporation in lactic acid bacteria, as described previously. This method is now being used to transfer genes from different sources into lactic acid bacteria.

A. Cloning Vectors

Many vectors have been constructed by combining nucleotide segments encoding desirable characteristics from different sources, such as the origin of replication, highly efficient promoters, multiple cloning sites, selective markers, and inducible systems. A vector should be stable and able to replicate in host cells. Generally, vectors that replicate by the theta mechanism are more stable than those that replicate by the sigma mechanism. Many small vectors have been constructed from small cryptic plasmids of lactic acid bacteria, such as pGK12 from pWV01. They are very efficient for cloning, transforming, and expressing genes in different lactic acid bacteria. Several shuttle vectors have also been constructed, which can be used to transfer and express genes between heterologous donors and recipient (e.g., pHPS9 in Figure 12.2). Because there are restrictions on the use of vectors carrying antibiotic markers to develop lactic acid bacterial strains for use in food fermentation, several food-grade vectors have been constructed. They carry food-grade selective markers, such as resistance to nisin, sucrose hydrolysis, and *ochre* suppressor, and help in selecting out the transformed cells.

For the efficient expression of the cloned genes, a vector should have proper transcription and translation signals upstream of the coding region. Both constitutive and inducible promoters have been integrated in vectors, which greatly enhance the expression of the cloned genes (e.g., the inducible *lac A* promoter and *his A* promoter). In addition, vectors that allow the isolation of secretion signals have been constructed. Their use has enabled the secretion of heterologous proteins as well as their anchorage to the cell wall of host cells.[3,4,8,9]

B. Metabolic Engineering

Many lactic acid bacteria are used to produce fermented foods and desirable by-products, because they can metabolize several carbohydrates, proteins, and lipids present in the raw materials used in the fermentation process. In most strains, the ability to produce a desirable characteristic in a fermented food or a valuable by-product is quite limited. Also, the possibility of producing a novel food or a by-product by using these natural strains is quite low. However, an understanding of the functions of many genes and their regulatory systems has helped conduct

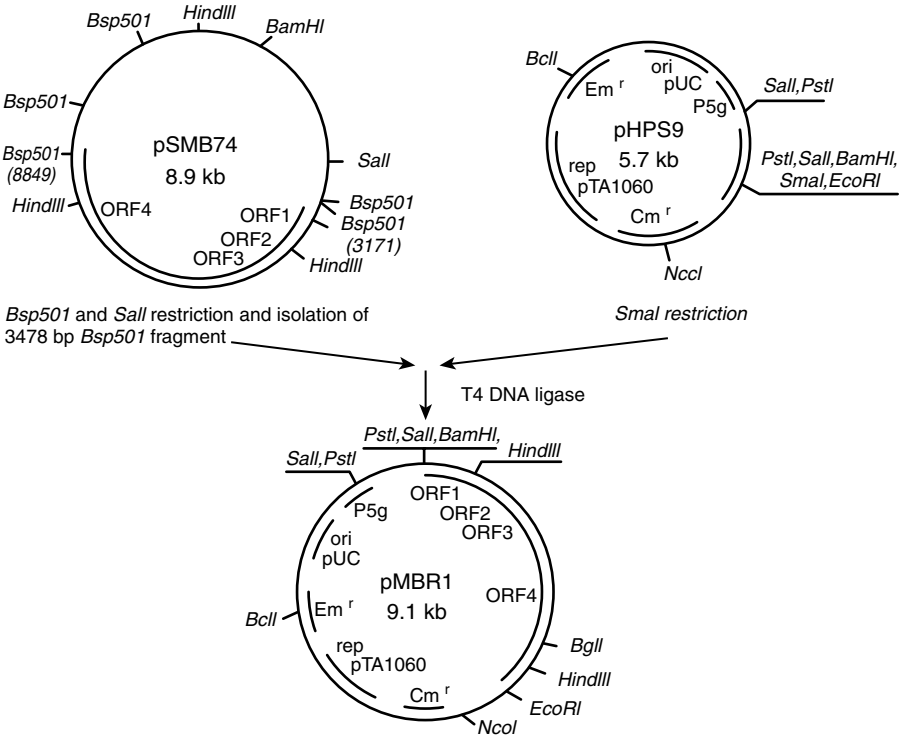


Figure 12.2 Construction of a recombinant plasmid. A 3.5-kb fragment carrying four open reading frames from pSMB74 was derived by digesting with *Bsp* 501 and cloned in the *Sma* 1 site of pHPS9 to produce the 9.1-kb pMBR1.0.

metabolic engineering in lactic acid bacteria to change the metabolic pathways, and, in some instances, to produce a higher level of by-products and better products. This approach has a great potential to produce unique bacterial strains, and many studies are being conducted in this field. Several examples are listed here.[10–12]

1. Mixed Acid Fermentation by Lac. lactis

Lac. lactis strains metabolize fermentable carbohydrates by the EMP pathway to produce mainly lactic acid. In the terminal step, pyruvate is reduced to lactate by lactose dehydrogenase (LDH), which is encoded by the *ldh* gene. When the *ldh* gene is inactivated by plasmid insertion, the LDH-negative strain reroutes pyruvate to produce acetate, formate, ethanol, and acetoin. Ethanol production is greatly increased by cloning the necessary genes from an ethanol-producing bacterium to the LDH-negative lactic acid bacteria. Similar results are also obtained by inactivating both *ldh* genes in a *Lab. plantarum* strain (which has two LDH enzymes).

2. L(+)-Lactic Acid Production

Lactic acid bacteria, such as *Lab. acidophilus, Lab. plantarum, Lab. fermentum, Lab rhamnosus, Ped. acidilactici,* and *Ped. pentosaceus,* that produce proportionately high quantities of lactic acid in the EMP pathway produce a mixture of both L(+)- and D(–)-lactic acid from pyruvate, because they contain both L- and D-lactate dehydrogenases. Because L(+)-lactic acid is produced in the body, it is preferred to D(–)-lactic acid as a food additive, and the strains mentioned are not preferred for commercial production of lactic acid for use in food. Because many of the species mentioned are also used to produce fermented foods, studies are being conducted to produce strains of these species that produce only L(+)-lactic acid. These species have the two genes, *ldh* L and *ldh* D, encoding the two LDHs. Strains of some of the species mentioned have been developed in which *ldh* D expressing D-LDH has been inactivated, enabling them to produce only L(+)-lactic acid.

3. Diacetyl Production by Lac. lactis

Diacetyl is associated with a pleasant butter aroma and is used in many nondairy products to give desirable characteristics. It is normally produced by some lactic acid bacteria, including the biovar diacetylactis of *Lac. lactis* species. Other *Lac. lactis* species and strains produce, if at all, very little diacetyl. Metabolic engineering has, however, enabled development of overproducing diacetyl strains of *Lac. lactis*. To develop this strain, first the *nox* gene (NADH oxidase) from a suitable source is cloned under the control of the nisin-inducible *nis A* promoter (NICE) in the *Lac. lactis* strain. In the presence of a small amount of nisin, the *nox* gene in the strain is overexpressed. In the next step, the gene encoding for α-acetolactate decarboxylase (ALDB, which converts α-acetolactate to acetoin, see Figure 11.5) is inactivated. This results in the accumulation of α-acetolactate that is produced from pyruvate. Under the overexpression of the *nox* gene and with inactivated ALDB, α-acetolactate is converted to diacetyl in high levels.

4. Alanine Production from Carbohydrates

Amino acids from proteins are generally used for growth and synthesis in lactic acid bacteria and do not accumulate in the cells or in the environment. However, by metabolic engineering, a strain of *Lac. lactis* has been developed that uses carbohydrate and ammonium sources to produce higher concentrations of alanine. The alanine dehydrogenase gene from a suitable source was cloned under the control of the NICE system. When the cloned vector was introduced in a LDH-negative strain and the strain was grown in the presence of a small amount of nisin and ammonium supplements, a large amount of carbohydrates was converted to L-alanine. Because L-alanine has a sweet taste, this strain can be used as a starter culture to produce fermented products with different, but agreeable, tastes.

5. Production of Mannitol and Other Polyols

Mannitol, a sugar alcohol, is produced by some strains of lactic acid bacteria, such as *Lac. lactis* and *Lab. plantarum*, in small amounts. It is produced by the reduction

of fructose-6-phosphate (in the EMP pathway) to mannitol-1-phosphate, which then is dephosphorylated by mannitol phosphate dehydrogenase (MPDH) to mannitol. However, 90% of it remains inside the cells. In contrast, *Leuconostoc mesenteroides* secretes most of the mannitol in the environment because it has an efficient mannitol transport system (it also produces high amounts of mannitol). Overproduction of mannitol by an LDH-deficient *Lac. lactis* strain has been achieved by metabolic engineering for overproduction of MPDH and low production of phosphofructokinase. By introducing the mannitol-transfer system from *Leu. mesenteroides*, the engineered *Lac. lactis* strain is able to excrete most of the mannitol in the environment. By similar techniques, *Lac. lactis* strains have been developed that are able to produce large amounts of sorbitol and tagatose. Mannitol, sorbitol, and tagatose (as well as L-alanine, discussed previously) can be used as low-calorie sweeteners in food.

6. Production of Folic Acid and Riboflavin

Many lactic acid bacteria, such as *Lac. lactis* and *Str. thermophilus*, while growing in milk and other fermented foods synthesize low levels of folate, but most of it is retained inside the cells. The multienzyme biosynthetic pathways of tetrahydrofolate from glutanyltriphosphate (GTP) in *Lac. lactis* have been identified. With this information, a strain of *Lac. lactis* has been developed that overexpresses the genes involved in the process. Initially, the engineered strain produces three times more folate than the wild strain does and excretes most of it in the environment. In the same manner, a *Lac. lactis* strain has been engineered that produces higher amount of riboflavin. This way the nutritional value of the fermented foods can be increased.

7. Enhancing Proteolysis by Cell Lysis

The desirable flavor of cheese is the result of proteolysis of milk proteins by extracellular and intracellular proteolytic enzymes of a starter culture during ripening. As the intracellular enzymes are released slowly (after death and lysis of cells of starter cultures during ripening), the process is relatively slow. Methods, including metabolic engineering, are being studied to enhance the ripening of cheese. In a metabolic engineering method, the lytic genes of bacteriophages are used to lyse the starter cells. This has been achieved by cloning the phage genes encoding for lysin and holin (cause cell lysis) under the nisin-induced promoter in *Lac. lactis*. In the presence of nisin, the cells lyse, releasing the proteinases and peptidase, which then help accelerate the ripening of cheese.

C. Protein Targeting

There is evidence now that many heterologous proteins, both from procaryotes and eucaryotes, can be expressed and produced in high levels in lactic acid bacteria by cloning the genes in suitable expression vectors and using existing or new secretory signals. The products can be excreted in the environment, attached on the cell wall and membrane, or even remain inside the cells. The possibilities are many and include many enzymes, antimicrobials, flavor compounds, and important bioactive

molecules for use as pharmaceuticals (immunity protein vaccine) and in agriculture (insecticides). Genes of many heterologous proteins have been cloned in *Lac. lactis* strains, such as chicken egg lysozyme, bovine prochymosine, α-amylase, pediocin (a bacteriocin), luciferase, interleukin 2, cholera toxin B, tetanus toxin C, and Cry 1A insect toxin. Many of the genes express at low to high levels, but a few do not express to the level of detection.[8,11]

1. Expression of Interleukin

A murine interleukin 2 (*mIL2*) expression vector, pL2MIL2, was constructed from pLET2N (originally developed from pLET2, the lactococcal T7 polymerase-based system). The plasmid, pL2MIL2, encodes the part of the *mIL2* gene that codes for the mature protein, fused with the lactococcal *usp* 45 secretory leader signal. *Lac. lactis* MG 1820 was transformed with this plasmid. When grown in an appropriate growth medium, the cells synthesized the USP 45-mIL2 fusion protein and secreted the mature mIL2 into the growth medium. The purified mIL2 was found to be biologically as active as the natural mIL2. By similar procedures, other murine interleukins (such as mIL-6 and mIL-10), murine interferon, and human interleukin have been produced by *Lac. lactis*.[13] Recent studies have shown that daily ingestion of *Lac. lactis* that can produce biologically active mIL-10 interleukin has cured and prevented enterocolitis in mice.[14]

2. Drug-Delivery System

Many lactic acid bacteria are normally present in the gastrointestinal (GI) system of humans and food animals and birds, and some of them have beneficial effects on the health and well being of the hosts (see Chapter 15). Currently, studies are in progress to anchor antigens of pathogens associated with enteric diseases in humans on the cell wall of suitable lactic acid bacteria. The live cells then can be used as a drug-delivery vehicle to deliver antigens to stimulate antibody production via the digestive tract. One method used to anchor proteins is to use *Lac. lactis* Prt P, which normally remains attached to the cell wall with its carboxy-terminal domain. The effectiveness of the carboxy-terminal cell wall anchor of *Staphylococcus aureus* protein A to the cell wall of *Lac. lactis* is also being investigated.

3. Production of Pediocin in Heterologous Hosts

The bacteriocin pediocin PA-1/AcH is produced by many strains of *Ped. acidilactici*. The molecule is translated as a 62-amino-acid prepediocin from which the 18-amino-acid leader peptide at the NH_2 terminal is enzymatically removed during membrane translocation through the ABC transporter system (Chapter 16). The 44-amino-acid pediocin is then released in the growth medium. The phenotype is plasmid linked and the structural gene with three other genes is arranged in an operon system, and the protein encoded by the last gene acts as the ABC transporter. The DNA fragment with all four genes has been cloned in several Gram-negative vectors and in a shuttle vector, and the host strains produce active pediocin molecules, generally at lower concentrations.

In a separate study, only the nucleotide segment of the matured pediocin (without the leader segment), was fused to the carboxy terminus of the maltose-binding protein (MBP), a secretory protein, and cloned in a Gram-negative vector. *Escherichia coli* transformed with the cloned vector secreted the chimeric MBP–pediocin by the Sec-dependent transport system in the growth media. The MBP–pediocin molecule, although much larger, retained the bactericidal property as that of natural pediocin. The pediocin operon has also been cloned in suitable vectors and transformed in *Lac. lactis* and *Str. thermophilus* strains, which produced pediocin. Generally, the production is low and the phenotype is unstable in the host in the absence of selective pressure such as antibiotics (the genes encoding the resistance to antibiotics are used as selective markers in the cloning vectors).[15]

D. Protein Engineering

Many studies are being conducted to change the amino acid sequences of proteins of lactic acid bacteria to determine the influence on the physical, chemical, and biological properties (structures and functions) of the proteins.

1. Production of Hybrid Prepediocin

Like the prebacteriocin molecules of lactic acid bacteria, prepediocin molecules have an 18-amino-acid leader segment at the NH_2 terminus and a 44-amino-acid segment at the carboxyl end. Following translation, the leader peptide directs the molecule for translocation through the ABC transporter. In one study, the promoter and nucleotide sequences of the leader segment of the pediocin structural gene were replaced with a similar segment associated with the production of the bacteriocin lactococcin A in a *Lac. lactis* strain. The structural gene was cloned in the same *Lac. lactis* strain, which produced propediocin with a different leader peptide but produced matured pediocin by using the lactococcin A-transport system. However, the level of production was very low, suggesting the important roles of leader peptides and specific transporters in high-level production of pediocin (Chapter 16).[15]

2. Amino Acid Variants of Pediocin

By random as well as site-directed PCR mutagenesis of nucleotides in the mature pediocin segment, many variant molecules have been isolated that have one or two different amino acids among the 44 amino acids. The variants have different levels of activity: some do not have detectable activity, many have reduced activity, and one has greater activity. These results indicate the importance of specific amino acid sequences of a bacteriocin for its normal level of activity.[15]

V. GENOME MAPPING AND SEQUENCING

Because of the importance of many lactic acid bacteria in the production of bioprocessed foods, food preservation and different food additives, enzymes and nutraceu-

ticals, maintenance of intestinal health, and as agents for drug-delivery systems, considerable research with many species of lactic acid bacteria has been conducted in the last 20 to 30 years. To understand the characteristics at the molecular level, for the last several years there have been efforts to sequence the complete genome (chromosome) of some strains of important lactic acid bacteria. Availability of new techniques and necessary facilities (equipment, computerized programs, and others) has greatly helped generate information in a relatively short time. These techniques have also helped sequence the genomes of many phages and prophages of lactic acid bacteria. The available information is briefly summarized here.

A. Lactic Acid Bacteria

As of 2002, complete genome sequences of five lactic acid bacteria have been completed and of 25 other important strains (of which 23 are lactic acid bacteria, including *Bifidobacteria*) are in progress.[16] These results have provided much important information, such as the size of each genome; number of ORFs a genome carries; and the frequency of prophages, IS-elements, and rRNA operons in a genome (Table 12.1). In addition, other genetic information, such as biosynthetic pathways of amino acids, vitamins, nucleotides, and polysaccharides; synthesis of bacteriocins; and sugar transport and metabolism systems, is now available for these strains. The *Lac. lactis* IL 1403 genome is the first to be completely sequenced.[17] The genome size is ca. 2.4 Mb and has 2310 ORFs with 138 potential regulators, 5 prophages, 6 different types of IS elements, and 6 rRNA operons. The genome encodes nucleotide sequences for *de novo* biosynthetic pathways of 24 amino acids, folate, riboflavin,

Table 12.1 Some Features of Sequenced Genome of Lactic Acid Bacteria[a]

Species/ Strains	Genome Size (Mb)	% GC	ORFs	Prophages	IS Element Types	rRNA Operons
Lac. lactis IL 1403	2.4	35.4	2310	5	6	6
Lab. plantarum WCFS 1	3.3	44.5	3050	2	2	5
Lab. johnsonii NCC 533	2.0	34.6	1857	0	6	6
Lab. acidophilus ATCC 700396	2.0	34.7	1979	3	3	6
Bif. longum NCC 2705	2.2	60.1	1730	Some	5	4

[a] Genome sequences of other lactic acid bacteria now being determined are *Lac. lactis* ssp. *cremoris* (3 strains); *Str. thermophilus* (3 strains); *Leu. mesenteroides* (1 strain); *Oen. oeni* (2 strains); *Ped. pentosaceus* (1 strain); *Bif. breve* (1 strain) and *longum* (1 strain); *Lab. delbrueckii* ssp. *bulgaricus* (3 strains); *Lab. casei* (2 strains), *helveticus*(2 strains), *rhamnosus* (1 strain), *brevis* (1 strain), *sake* (1 strain), and *gasseri* (1 strain); *Pro. freudenreichii* (1 strain); and *Bre. linen* (1 strain).

Source: Adapted from Klaenhammer, T. et al., *Ant. van Leeuwen.,* 82, 29, 2002.

purines and pyrimidines, complete PTS-sugar transport systems, and partial components of aerobic respiration. The genome appears to have a fluid structure and is able to undergo changes through point mutation, DNA rearrangements, and horizontal gene transfer. The genome organization has resulted from the evolutionary pressure to enable the species to grow optimally in a nutritionally rich medium.[18,19]

As the genome sequence of different species and strains of lactic acid bacteria become available, the information can be used to compare and understand various important characteristics of lactic acid bacteria, such as determining the evolutionary relationship among the species and strains in various genera, functions of each gene and its essential and nonessential nature, and functions of the noncoding region on the regulation and expression of a gene or an operon system. This information can then be used to modulate gene expression and efficiently conduct metabolic engineering to develop new strains for use in producing novel fermented products and important by-products.[16–19]

B. Bacteriophages

The genomes of 20 important temperate and virulent bacteriophages of lactic acid bacteria (from genera *Lactococcus, Lactobacillus,* and *Streptococcus*) have been completely sequenced. Most have genomes ranging from 20 to 55 kb. The information has helped locate and understand the functions of many ORFs and regulatory regions. Some of them are the genes involved in packaging phage DNA in the heads, genes associated with lysis of host cells, and genes controlling the lysogenic and lytic cycles of a phage. An understanding of these factors has helped develop phage-resistant lactic acid bacterial strains for use in food fermentation and to improve acceptable characteristics of some fermented foods (e.g., accelerated cheese ripening) and transferring and improving expression of genes in lactic acid bacteria.[20] Several methods of developing phage-resistant starter-culture bacteria are listed in Chapter 13.

Analyses of genomes of lactic acid bacteria reveal the presence of many prophages. Although they pose a metabolic burden to host cells and if induced may lyse the host cells, it is surprising that the cells carry them. However, the cells carrying the prophage are resistant to attack by temperate phage and will be thus dominant in the population. In addition, a mutation in the prophage induction system will enable the cell to maintain resistance without being lysed. This could lead to development of phage-resistant lactic acid bacterial strains.[20]

C. The *lac* and *las* Genes

The genes associated with metabolism of lactose to lactic acid in different lactic acid bacteria are located in at least two operon systems, and are grouped as *lac* genes and *las* genes. The *lac* genes are associated with the transport and hydrolysis of lactose to two hexoses, and partial metabolism of some hexoses, whereas the *las* (lactic acid synthesis) genes are involved in the production of lactic acid.[17,21]

The *lac* genes, depending on a species and strain, can be located either on a chromosome or on a plasmid. In most *Lac. lactis* strains, they are plasmid linked,

but in *Lab. delbrueckii* ssp. *bulgaricus* and *Lab. helveticus* they are on the chromosomes. Whereas in *Lac. lactis* they are inducible (also in *Lab. acidophilus*), in the two *Lactobacillus* species they are constitutive. Limited studies have revealed *lac* genes to be plasmid linked in *Leu. lactis* and chromosomally linked in *Str. thermophilus*.

In the *Lac. lactis* strains, lactose fermentation usually includes its transportation by the PEP-PST system as lactose phosphate, hydrolysis by phospho-β-galactosidase to glucose and galactose-6-phosphate, and conversion of galactose-6-phosphate to tagatose-6-phosphate and then to two triosephosphates before entering the EMP pathway (see Chapter 11). Glucose is also converted to triosephosphate in the EMP pathway. Triosephosphates are finally converted to lactic acid. In *Lab. delbrueckii* ssp. *bulgaricus*, lactose is transported (along with a proton) by lactose permease and hydrolyzed by β-galactosidase to glucose and galactose. Glucose is metabolized through the EMP pathway, whereas galactose is first metabolized by the Leloir pathway to glucose-1-phosphate before entering the EMP pathway. In *Str. thermophilus*, following transport of lactose by lactose permease and hydrolysis by β-galactosidase to glucose and galactose, glucose is metabolized by the EMP pathway; in most strains, galactose is excreted into the environment.

The plasmid-linked *lac* genes in *Lac. lactis* strains have been characterized, and the function of each gene has been determined. A total of eight genes are arranged in an operon system and designated as *lac ABCDFEGX*. The four genes, *lac ABCD*, encode the three enzymes in the tagatose-6-phosphate pathway: the *lac AB* is involved in the conversion of glucose-6-phosphate to tagatose-6-phosphate, *lac C* in the conversion of tagatose-6-phosphate to tagatose-1,6-diphosphate, and *lac D* in the conversion of the latter to glyceroldehyde-3-phosphate and dihydroacetone phosphate. The lac *FE* genes encode the PEP-PTS and the *lac G* encodes the hydrolysis of lactose-6-phosphate. The function of *lac X* is not known. The promoter of the operon is located upstream of *lac A,* and the regulator gene (*lac R*) is located immediately upstream of the promoter. It encodes the repressor protein that regulates the expression of *lac* operon, which is inducible. The arrangement, location, and regulation of *lac* genes in other lactic acid bacteria have not yet been properly identified.[17,21]

The *las* genes, or the genes encoding for lactic acid synthesis, have been identified and characterized on the chromosome of *Lac. lactis*. Five genes are involved for encoding the enzymes necessary in the process: *pfk*, for phosphofructokinase; *pyk*, for pyruvate kinase; *ldh*, for L-(+)-lactate dehydrogenase; *tpi*, for triosephosphate isomerase, and *gap*, for glyceraldehyde-3-phosphate dehydrogenase. The three genes, *las pfk, las pyk,* and *las ldh* are organized in an operon (the *las* operon) with the promoter located upstream of *las pfk* and a terminator located downstream of *las ldh*. The genes *las tpi* and *las gap* are located separately on the chromosome and each is expressed as a monocistronic gene.

The five genes are highly biased in their codon usage, suggesting they are strongly expressed in *Lac. lactis*. Limited results have indicated that the expression of the three genes in the *las* operon is under a genetic regulatory mechanism, which limits their expression and the level of lactic acid production.[17,21]

VI. CONCLUSION

The importance of many strains of lactic acid bacteria in the fermentation of diverse types of food, production of different food additives, use as probiotics, and application to deliver drugs has necessitated the understanding of the molecular basis of these desirable characteristics. With a modest beginning in early 1970s, the research activities on genetics of lactic acid bacteria have exploded. Currently, studies on genome sequence of lactic acid bacteria are generating important information on the fluid nature of the DNA that can change with environment. Comparative genome analysis, with more information, will enable researchers to determine the minimum genetic information and the arrangements of genetic codes necessary to design a unique starter-culture strain for a specific use. Production of starter cultures and problems associated with bacteriophages are discussed in Chapter 13.

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QUESTIONS

1. Discuss the important characteristics of plasmids in lactic acid bacteria.
2. Discuss the characteristic differences between large and small plasmids in lactic acid bacteria.
3. List five commercially important phenotypes that are plasmid linked in lactic acid bacteria and discuss their importance in relation to plasmid stability.
4. Define and list the advantages and disadvantages of the following methods used in gene transfer: transduction, transfection, transformation, and protoplast fusion in lactic acid bacteria.
5. Describe the process involved in conjugal transfer of DNA in lactic acid bacteria. What are the advantages and disadvantages of this method?
6. Describe the technique involved in electrotransformation of DNA in lactic acid bacteria. Discuss the advantages of transferring DNA by this method over the other methods in lactic acid bacteria.
7. Define the following terms and briefly discuss their importance in lactic acid bacteria: cryptic plasmid, plasmid replication, electrotransformation, IS-elements, and transposons.
8. Define the term *metabolic targeting*. With a proper example, explain how metabolic targeting in lactic acid bacteria can be used to produce diacetyl, L(+) lactic acid, and folic acid in strains that have low ability to produce them.

9. Explain with one example the importance of (a) protein targeting and (b) protein engineering research in lactic acid bacteria.
10. Explain how the information of genome sequence of lactic acid bacteria is helpful in their application in strain development.
11. List three important applications that can be developed from the genome sequences of phages of lactic acid bacteria.
12. Briefly discuss the functions of (a) *lac* genes, (b) *las* genes.

CHAPTER 13

Starter Cultures and Bacteriophages

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I. INTRODUCTION

Starter culture is a generic term and has changed its meaning over the years. Currently, it means a selected strain of food-grade microorganisms of known and stable metabolic activities and other characteristics that is used to produce fermented foods of desirable appearance, body, texture, and flavor.[1] Some starter cultures are also used to produce food additives, as probiotics, and for drug delivery. Toward the end of the 19th century, the term meant inoculating a small amount of fermented (sour) cream or milk (starter) to fresh cream or milk to start fermentation in the

production of butter and cheese, respectively.[2] This process was found to give better products than those produced through natural fermentation of the raw materials. These starters were mixtures of unknown bacteria. Processing plants started maintaining a good starter by daily transfer (mother starter) and produced product inoculum from these. However, the bacteriological makeup of these starters (types and proportion of the desirable as well as undesirable bacteria) during successive transfers was continually susceptible to changes as a result of strain dominance among those present initially, as well as from the contaminants during handling. This introduced difficulties in producing products of consistent quality and from product failure due to bacteriophage attack of starter bacteria. Some private companies started supplying mixed cultures of unknown bacterial composition for cheese manufacture both in the U.S. and in Europe. Subsequently, the individual strains were purified and examined for their characteristics, and starter cultures with pure strains were produced by these commercial companies. Initially, such starter cultures were developed to produce cheeses. Currently, starter cultures for many types of fermented dairy products, fermented meat products, some fermented vegetables, fermented baking products, for alcohol fermentation, and for other purposes (especially with genetically modified organisms, GMOs) are commercially available. In this chapter, a brief discussion on the history, current status, bacteriophage problems, and production of concentrated cultures is presented.

II. HISTORY

Initial development of starter cultures resulted from the need and changes in the cheese industry. Before the 1950s, small producers were producing limited amounts of cheese to satisfy local consumers. A plant used to maintain a bottle of mother culture by daily transfer, which it received from a culture producer or another neighboring processor. From the mother culture, the plant used to make bulk culture through several transfers to meet the inoculation volume (to meet the need of 1 to 2% of the volume of milk to be processed for cheese). These starters were a mixture of undefined strains of bacteria, and it was difficult to produce a product of consistent quality. There were also problems with starter failure from bacteriophage attack. To overcome the problem of quality, the single-strain starter (a desired strain isolated from a mixture) was introduced. Good sanitation and newly designed processing equipment were introduced to overcome phage problems.[1,2]

Since the 1950s, large cheese operations have replaced the small producers. They needed products of consistent quality and could not afford to have too many starter failures from phage attack. Starter-culture producers developed single-strain cultures and supplied these in dried form to the cheese processors, who, in turn, used them to produce mother cultures and bulk cultures. To overcome phage problems, rotation of strains (such as using different strains each day) to prevent the buildup of a particular phage, as well as multiple-strain cultures (if one strain is killed by a specific phage, another will work), were practiced. Later, defined media to produce bulk cultures that reduced phage attack were introduced. Even then, daily production of large amounts of bulk cultures (some cheese processors were handling more than

1,000,000 gal of milk daily and needed more than 10,000 gal of bulk cultures) and maintaining a large number of defined bacterial strains for use in rotation or multiple-strain cultures (some were keeping 30 or more strains) and using them in proper combinations (so that they are compatible) demanded defined and large facilities, expert microbiologists, and a large crew for the operation. This was partially overcome with the introduction in the 1960s of frozen concentrate cultures that could be shipped by air from culture producers to cheese processors in dry ice and could be used directly to produce bulk cultures. This, along with the availability of several types of phage inhibitory media (PIM) to produce bulk cultures, helped cheese processors overcome the cost and labor necessary to maintain a microbiological laboratory and a large number of starter strains. These dairy-based media contain a high concentration of phosphate to chelate calcium in milk and thus make the divalent cation unavailable for the adsorption of phages to the bacterial cells and cause infection. To obtain high cell density by maintaining a high pH, the media had either internal or external pH control systems. Subsequently, frozen concentrated cultures, containing $10^{11–12}$ cells/ml were introduced; they could be directly inoculated into milk in the cheese vat (direct vat set, DVS), eliminating the need to produce bulk starter by a cheese processor.

From the 1970s, the popularity of several types of fermented dairy products (particularly buttermilk and yogurt), as well as fermented sausages, some fermented ethnic products, and fermented health products, stimulated their production by large commercial processors. To meet their need, different types of frozen concentrated cultures for direct inoculation into the raw materials were developed.

Efforts have been made to produce freeze-dried concentrated cultures. Dried cultures can eliminate the bulk problem in transporting frozen concentrated cultures in dry ice as well as their accidental thawing, which would thus prevent their use. Dried cultures can also be used directly for product manufacture or can be used to produce bulk starters. However, many strains do not survive well in the dried state. Thus, their use as dried cultures in large commercial operations has been limited. They are available in small packages for use directly by the small processors of fermented foods or for use to produce bulk cultures before inoculation in the raw material.

A fairly recent advance has been the availability of custom-designed starter cultures to meet the specific needs of a food processor. An understanding of the genetic basis of some important desirable traits, as well as traits for phage attack inhibition in starter cultures, has helped produce designer cultures.

III. CONCENTRATED CULTURES

In controlled fermentation of food, a starter is added to a raw material at a level of ca. $10^{6–7}$ live cells/ml or live cells/g for the fermentation to proceed at a desired rate. In the conventional process, a bulk culture with ca. $10^{8–9}$ cells/ml needs to be inoculated at ca. 1% level to the raw material. A cheese processor who uses 100,000 gal or more milk per day needs 1000 gal or more bulk starter daily. This large volume is produced from mother cultures through several intermediate transfers, involving more handling at the processing facilities and possible introduction of phages, as

the phages are more abundant in the processing environment (Figure 13.1). In contrast, in the production of concentrated culture, most handling is done by culture producers under controlled environmental conditions, thereby minimizing phage problems. The strains are grown in suitable media to obtain high cell density (some to more than 10^{10}/ml). The cells are harvested by centrifugation and resuspended in a liquid at a concentration of ca. 10^{12} cells/ml. A 360-ml frozen concentrate culture in the DVS system can be added to 5000-gal milk vat to get the desired initial concentrations of viable cells (10^{6-7} cells/ml). The suspending medium contains cryoprotective agents to reduce cell death and injury during freezing and thawing. The cells in a mixture in metal containers are frozen, either by using dry ice–acetone (–78°C) or liquid nitrogen (–196°C), and stored at that temperature. They are transported to processors, preferably by air, in Styrofoam™ boxes with sufficient amounts of dry ice. A processor stores the containers at –20°C or below and uses it within the time specified by the culture producer. Just before use, a container is thawed in warm (45°C) potable water and added to the raw material. The directions for use of these cultures are supplied by culture producers.[3]

To produce freeze-dried concentrates, the liquid cell concentrates are shell frozen (in thin layers or small droplets) in either dry ice acetone or in liquid nitrogen and then dried under vacuum. The dried materials are packed in plastic bags under vacuum and stored at –20°C or at refrigerated temperature. They are transported to processors for quick delivery, either at ambient temperature or in boxes containing ice packs. A processor stores the dried cultures at –20°C or in a refrigerator and uses them within the specified time. Just before use, the dried culture is mixed with warm water (preferably boiled and cooled or sterile) and added to the raw materials.[3]

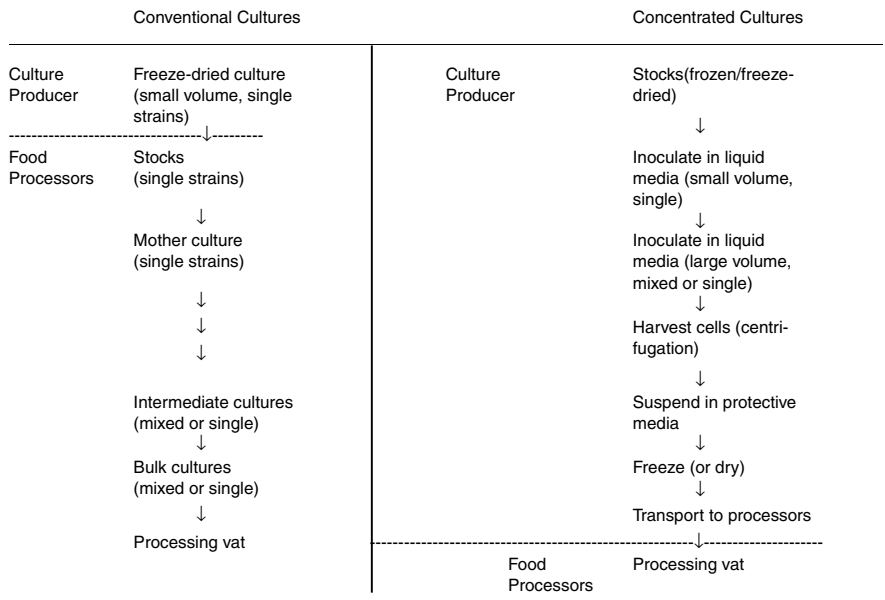


Figure 13.1 Production steps and use of conventional and concentrated cultures and changes in culture handling by culture producers and food processors.

IV. STARTER-CULTURE PROBLEMS

A. Strain Antagonism

In mixed-strain cultures, in which a starter culture contains two or more strains, dominance of one over the others under a given condition can change the culture profile quickly. Dominance can result from optimum growth environment or production of inhibitory metabolites (e.g., bacteriocins). This can affect product quality and increase starter failure through phage attack. Culture producers test the compatibility between desirable strains and develop mixed-strain cultures with only the compatible strains to avoid strain antagonism.[4]

B. Loss of a Desired Trait

A strain carrying a plasmid-linked desired trait can lose the trait during storage, subculturing, and under some growth conditions.[4] Physical and chemical stress and long freezing can also result in loss of a trait. Genetic studies are being conducted to understand the mechanisms of the stability of these traits. Several strains with better stability of some traits have been developed and are being used. In the future, more such strains will be available.

C. Cell Death and Injury

The effective use of frozen and freeze-dried concentrated cultures, especially for direct use (such as DVS cultures), depends on two important characteristics: (1) cultures need to have large numbers of viable cells and (2) cells should have a short lag phase so that they can start multiplying very quickly. The cells can be exposed to adverse physical and chemical environments (or stresses) that can reduce survival, growth, and metabolism.[5] Freezing and thawing, and freeze-drying and rehydration, are known to cause cell damage, leading to cell death and cell injury (Chapter 9). This can lead to situations wherein a concentrate culture fails to meet the needs of initial viable cell concentrations (10^{6-7} cells/ml or cells/g of raw materials) and the relatively short lag in the fermentation process. Culture producers have been successful in reducing cell damage by using cryoprotectants in the suspending menstrua and freezing the cells rapidly at a very low temperature. Some of the causes of cell viability loss are thawing and refreezing, thawing long before using, mixing thawed cultures with or rehydrating dried cultures in concentrated solutions of other constituents (such as curing salt and spice mixtures used in sausage fermentation), and long storage at –20°C or at higher temperatures.[4] Many of these occur at the product processing environment due to lack of knowledge of the people handling the cultures. For the best performance of concentrated cultures, directions from culture producers need to be strictly followed.

D. Inhibitors in Raw Materials

Milk can contain either antibiotics, given to the animals to treat some infections (mastitis), or sanitizers (from the equipment).[4] Meat can contain ingredients used to

make fermented sausages, such as phosphate or nitrite, and sanitizers from equipment. These factors can prevent or reduce the growth of starters.

E. Bacteriophages of Lactic Acid Bacteria

The role of bacteriophages (or phages) in starter-culture failure in food fermentation has been recognized for a long time. A short discussion on their life cycle that results in starter failure, as well as some current practices used to overcome phage problems in food fermentation, is presented here.[6–10]

1. Morphology and Characteristics

Bacteriophages are filterable viruses of bacteria widely distributed in the environment, especially in food fermentation environments. A phage contains several proteins (that make the head, tail, tail fiber, and contractile sheath) and DNA, which can be linear or circular, double stranded of ca. 20 to 55 kb in size. The double-stranded DNA molecule is packed in the head, which can be round or hexagonal (prolate or isometric, respectively).

Several schemes have been proposed at different times to classify the bacteriophages of lactic acid bacteria. Currently, a scheme is used in which the family, group, and morphotype are classified together. The three families *Myoviridae, Siphoviridae,* and *Podoviridae* are included in three groups, A (with contractile tails), B (with noncontractile long tails), and C (with noncontractile short tails), respectively. They can have three morphotypes, namely 1 (with small isometric heads), 2 (with small prolate heads), and 3 (with large prolate heads), respectively. *Lac. lactis* ssp. *lactis* and ssp. *cremoris* appear to have many wide varieties of phages. They have been divided into 12 species, of which 3 are in the *Siphoviridae* family and are important because of their virulent nature. The 3 in the *Siphoviridae* family are 936 species (all are virulent with small isometric head; e.g., sk1), c2 species (all are virulent with prolate head; e.g., c2), and P335 species (have both virulent and temperate phages and small isometric heads; e.g., TP901-1). Genome sequence analysis indicates that the species in the same family as well as those in different families vary considerably. In contrast, the genome analysis of phages of *Streptococcus thermophilus* (e.g., sfi 19, sfi 11) indicates a fairly good homology. Phages of several species of *Lactobacillus* have been identified and genome sequences of some species and strains are now available, namely for *Lactobacillus delbrueckii* (LL-H), *Lab. plantarum* (phi-gle), *Lactobacillus johnsonii* (Lj 965), *Lab. gasseri* (adh), and *Lab. casei* (a2). Very few phages of *Leuconostoc* and *Oenococcus* have been isolated, and none have been isolated from *Pediococcus*.[6]

2. Life Cycle

A phage cannot multiply by itself in food. Instead, it attaches (needs Ca^{2+} for adsorption) with its tail on the surface of a bacterial cell (specific host) and injects its DNA inside the cell cytoplasm. If it is a lytic phage, the bacterial cell produces a large number of copies of the phage DNA and phage proteins. Following

assembly of the proteins and phage DNA to produce mature phages, the bacterial cell lyses, releasing the phages (as many as 200) in the environment (Figure 13.2). They, in turn, attack other bacterial cells. These are lytic phages. Their growth cycles (lytic cycle) take ca. 20 to 30 min. There are other phages (called temperate phages) that are nonlytic and their DNA, following injection into the cytoplasm, is integrated with bacterial DNA. The phage DNA (called prophage) is carried by a bacterial cell DNA (lysogeny state), and as a bacterial cell multiplies, the prophage also multiplies without showing the presence of the phage in the bacterial strain (lysogenic strain). As long as the host cell carries the prophage, it is immune to attack by the same phage. Recent genome studies have shown that the DNA of a host strain can have five or more types of prophages.[6] However, a prophage can be induced by a physical (such as UV) or a chemical (such as mitomycin C) agent, causing the phage DNA to separate out of bacterial DNA and resume the lytic cycle (Figure 13.2).

3. Host Specificity

Bacteriophages against many species of *Lactococcus, Streptococcus, Leuconostoc,* and *Lactobacillus*, and *Oenococcus oenos* that are currently used in food fermentation have been discovered. The phages are host specific, and there can be one specific host (strain) for a specific phage to several related strains for a phage. A bacterial strain can also be the host of many different types of phages. A bacterial strain can have restriction enzymes that can hydrolyze and destroy the DNA of a phage. A phage can be lytic or temperate. All phages require Ca^{2+} for their adsorption on the cell surface of lactic cultures.

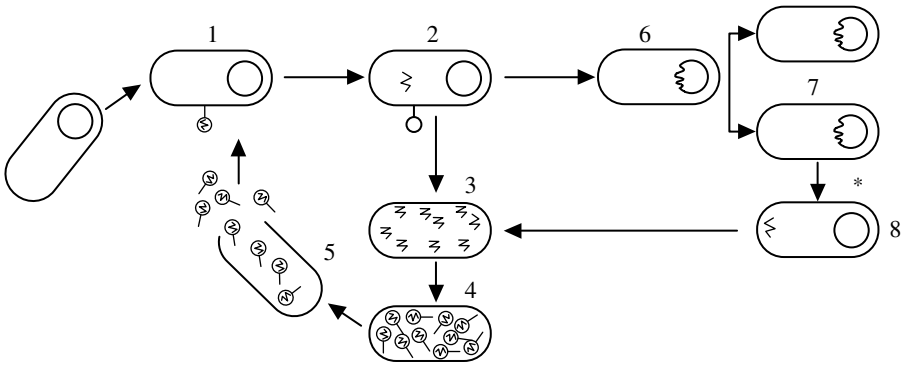


Figure 13.2 Schematic presentation of the lytic cycle and lysogenic cycle of bacteriophages in bacteria: (1) Adsorption of a phage on the bacterial cell wall. (2) Injected phage DNA in bacterial cell. (3–5) Lytic cycle by lytic phage showing formation of phage DNA (3) and mature phages (4) in the cell and release of phages following lysis of cell (5). (6–8) Lysogenic cycle by a temperate phage showing integration of phage DNA in bacterial cell DNA (6), division of phage DNA during bacterial cell division (prophage; 7), release of phage DNA because of activation (*; 8), causing lysis of cell (3 to 5).

4. Control Methods

Following the discovery of bacteriophage-related starter failure in food fermentation, steps were developed to reduce phage contamination with starter cultures.[7,9,10] These include proper sanitation to reduce phage buildup in the processing facilities, both during bulk starter preparation and product fermentation; use of phage-insensitive media; rotation of strains; and use of mixed strains to reduce the buildup of a particular phage to a level that causes starter failure. Subsequently, phage-resistant starter strains were developed. Initially, by growing a sensitive bacterial strain in the presence of a specific lytic phage, cells that were not killed by the phage were isolated and supplied to processors. Recent studies have indicated that by genetic techniques, a bacterial strain can be made insensitive to one or more phages. These include modifying the genetic makeup of a starter strain to inhibit phage adsorption, destroying phage DNA by restriction enzyme systems of cells, or aborting the phages before lysis. By combining these traits through genetic manipulation, a strain can be developed that is resistant to several phages. Current studies on genome analysis of lactic acid bacteria and bacteriophages will help develop phage-resistant starter strains (see Chapter 11).

V. YEAST AND MOLD CULTURES

Specific strains of yeast cultures (e.g., *Saccharomyces cerevisiae*) used to leaven dough in bakery products, and to produce alcohol in beer, wine, or distilled liquor, have been developed. These yeasts are produced by culture producers as well as by processors. Culture producers grow the yeast in suitable media, concentrate the cells, and supply in frozen or dried form.

Molds used as starter in some products are also available from culture producers. The strains used should not produce mycotoxins. The molds are grown on the surface of a liquid or solid (bread) media until they sporulate. The spores are collected, dried in powder form, packaged, and supplied to processors.

VI. CONCLUSION

Isolation and identification of microorganisms associated with food fermentation have helped the use of specific species and strains in pure culture for controlled fermentation. These starter cultures are currently produced by commercial culture producers for use by food-processing companies directly to start fermentation of raw materials. This has also helped to reduce product loss associated with culture failure, notably from phage attack. Development of phage-resistant starter cultures has also helped overcome the problem. Current studies on genome sequence of lactic acid bacteria and their bacteriophages will be useful in the future development of bacterial strains for their efficient use in food fermentation. The use of starter culture in food fermentation is covered in Chapter 14.

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QUESTIONS

1. Define starter culture.
2. Briefly list the major advances in starter-culture technology since the 1950s.
3. How are concentrate starter cultures prepared? How does this technology differ from the making of bulk starters?
4. Briefly describe the method used in developing a phage-insensitive medium for the manufacture of bulk starters.
5. List the problems in starter cultures that can affect their performance. What difficulties can occur because of thawing and refreezing a starter culture?
6. Define the terms lytic phage, temperate phage, prophage, lysogeny, and lytic cycle of bacteriophages.
7. Give an example of bacteriophages of each: *Lactococcus, Streptococcus,* and *Lactobacillus*.
8. How can starter strains resistant to bacteriophages be produced?

CHAPTER 14

Microbiology of Fermented Food Production

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I. INTRODUCTION

At the dawn of civilization humans recognized, probably by accident, that under certain circumstances, when raw foods from plant and animal sources were stored for future consumption, they might change to different but desirable products with longer storage stability. The possibility of such an event occurring might have been after they learned to produce more foods than they could consume immediately and thus needed storage. It was probably the period during which they learned agriculture and animal husbandry, as well as making baskets and pottery to store the excess products. On this basis, one can assume that fermented food probably originated ca. 7000 to 8000 B.C. in the tropical areas of Mesopotamia and the Indus Valley. Subsequently, other civilizations also produced fermented foods from different raw materials, particularly to preserve those that were seasonal and thus available in abundance only for a short harvesting period. Fermented milk products, alcoholic beverages from fruits and cereal grains, and leavened breads became popular among the early civilizations in the Middle East and in the Indus Valley and later among the Egyptians, Greeks, and Romans.[1]

Currently, more than 3500 different fermented foods are consumed by humans worldwide; many are ethnic and produced in small quantities to meet the needs of a group in a particular region. Some are, at present, produced commercially, and only a few are produced by large commercial producers. Production by large producers is now on the rise.

At present, there is interest in consumption of many types of fermented foods other than cheese, bread, pickles, and alcoholic beverages. One reason for this increase is consumer interest in natural and healthy foods, which fermented foods have been thought to satisfy. Even countries in which many types of fermented foods have been consumed for a long time, but mostly produced in small volumes, have started commercially producing some products in large volumes. It is anticipated that in the future, consumption of many fermented foods will increase worldwide.[1]

II. GENERAL METHOD OF PRODUCTION

The production of a fermented product has two related, yet separate, aspects, one involving the importance of metabolic activities of microorganisms during

fermentation and storage of the product and the other the parameters used during processing and storage of the product. In this chapter, the microbiological aspects are presented. Processing aspects are generally taught in a food-processing course and are thus covered only in brief here.

Fermentation involves exposing the raw or starting food materials to conditions that favor growth and metabolism of specific and desirable microorganisms. As the desirable microorganisms grow, they utilize some nutrients and produce some end products. These end products, along with the unmetabolized components of the starting materials, constitute the fermented foods having desirable acceptance qualities, many of which are attributed to the metabolic end products.

A. Raw (or Starting) Materials

A large number of raw materials from plant and animal sources are used to produce fermented foods. These include milk (from cows, buffalo, sheep, goats, and mares), meat (beef, pork, lamb, goat, and fowl), fish (many types), eggs (chicken and duck), vegetables and vegetable juices, many fruits and fruit juices, cereal grains, tubers, lentils, beans, and seeds. Some are used in combination.

B. Microorganisms Used

Many desirable species and strains of bacteria, yeasts, and molds are associated with fermentation of foods. Depending on a product, fermentation may be achieved by a single predominating species and strain. However, in most fermentations, a mixed population of several bacterial species and strains, or even bacteria and yeasts or bacteria and molds, is involved. When a fermentation process involves a mixed population, the members should not be antagonistic toward one another; rather, they should preferably be synergistic. Maximum growth of a desirable microorganism and optimum fermentation rate are dependent on environmental parameters such as nutrients, temperature of incubation, oxidation–reduction potential, and pH. In the fermentation process, if the different species in a mixed population need different environmental conditions (e.g., temperature of growth), a compromise is made to facilitate growth of all the species at a moderate rate. Depending on a raw or starting material and a specific need, carbohydrates (dextrose in meat fermentation), salts, citrate, and other nutrients are supplemented. In some natural fermentations, several species may be involved for the final desirable characteristics of the product. However, instead of growing at the same time, they appear in sequence, with the consequence that a particular species predominates at a certain stage during fermentation. But analyzing the final product to isolate the species involved in fermentation of such a food does not give the right picture. Instead, samples should be analyzed at intervals to determine predominant types at different times and to know the sequences in their appearance. Finally, some minor flora (secondary flora) can be present in a very low level in a raw material and the final product and might not be detected during regular analysis. However, they may have important contributions for the desirable characteristics, particularly some unique aroma, of the product.

C. Fermentation Process

Foods can be fermented in three different ways, based on the sources of the desirable microorganisms: natural fermentation, back slopping, and controlled fermentation.

1. Natural Fermentation

Many raw materials used in fermentation (usually not heat treated) contain both desirable and associated microorganisms. The conditions of incubation are set to favor rapid growth of the desirable types and no or slow growth of the associated (many are undesirable) types. A product produced by natural fermentation can have some desirable aroma resulting from the metabolism of the associated flora. However, because the natural microbial flora in the raw materials may not always be the same, it is difficult to produce a product with consistent characteristics over a long period of time. Also, chances of product failure because of growth of undesirable flora and foodborne diseases by the pathogens are high.

2. Back Slopping

In this method, some products from a successful fermentation are added to the starting materials, and conditions are set to facilitate the growth of the microorganisms coming from the previous product. This is still practiced in the production of many ethnic products in small volumes. Retention of product characteristics over a long period may be difficult because of changes in microbial types. Chances of product failure and foodborne diseases are also high.

3. Controlled Fermentation

The starting materials (may be heat treated) are inoculated with a high population (10^6 cells/ml or more) of a pure culture of single or mixed strains or species of microorganisms (starter culture). Incubation conditions are set for the optimum growth of the starter cultures. Large volumes of products can be produced with consistent and predictable characteristics each day. Generally, there is less chance of product failure and foodborne diseases. However, there may be no growth of desirable secondary flora. As a result, a product may not have some delicate flavor characteristics.

As indicated before, worldwide there are more than 3500 types of fermented foods. Different methods have been used to arrange them in several major groups. One such method divides fermented foods in nine groups and is presented in Table 14.1.

As it is beyond the scope of this textbook to describe even a few from each group, microbiological criteria of only several fermented dairy, meat, and vegetable products are briefly discussed here to understand the methods involved in controlled and natural fermentation. For more detailed information, books on fermentation of different food groups can be consulted, some of which have been used as references in this chapter.

Table 14.1 Fermented Food Groups and Examples

Food Groups	Examples
Dairy products	Cheeses, yogurt, buttermilk, sour cream, dahi, kumiss, kefir, acidophilus milk
Meat products	Salami, pepperoni, chorizo, Thüringer, sausage, pickled meat, nahm
Cereal products	Breads, pancake, crackers, pizza, nun, idli, dosa, sour rice, miso
Fruits and vegetable products	Pickled fruits, pickled vegetables, olives, sauerkraut, kimchi, achar
Legume products	Tofu, fermented soymilk, tempe, soy sauce, koji, mizo, natto, papadam
Fish products	Bagoong, fish sauces, pickled fish, tarama, paak, mamoni, izushi
Beverages	Beer, wine, distilled spirits, coffee, cocoa, tea
Starch crop products	Fermented products from potato, cassava, sweet potato, bananas, plantains, etc.
Miscellaneous products	Fermented eggs, ghee (from fermented cream), vinegar, red palm oil, bongkrek, dage

Source: Adapted from Campbell-Platt, G., *Fermented Foods of the World*, Butterworths, Boston, 1987.

III. FERMENTED DAIRY PRODUCTS

Fermented dairy products can be broadly divided into two groups: fermented milk products and cheeses. In fermented milk products, all the constituents of the milk are retained in the final products, with the exception of those partially metabolized by the bacteria. In cheeses, a large portion of milk constituents is removed in whey to obtain the final products.

A. Milk Composition and Quality

The growth of desirable microorganisms and the quality of a fermented dairy product are influenced by the composition and quality of the milk used in a fermentation process. Cow's milk contains approximately 3.2% protein, 4.8% lactose, 3.9% lipids, 0.9% minerals, traces of vitamins, and ca. 87.2% water. Among the proteins, casein in colloidal suspension as calcium caseinate is present in higher amounts than the other two soluble proteins, albumin and globulin. Lactose is the main carbohydrate and is present in solution, and lipids are dispersed as globules of different sizes in emulsion (fat in water). Minerals are present in solution and as colloid with casein. Water-soluble vitamins are present in aqueous phase, whereas fat-soluble vitamins are present with the lipids. The solid components (ca. 12.8%) are designated as total solids (TS), and TS without lipids is designated as solid-not-fat (SNF; ca. 8.9%). The whey contains principally the water-soluble components, some fat, and water.

The growth of desirable microorganisms can be adversely affected by several components that are either naturally present or have entered in the milk as contaminants. The natural antimicrobials are agglutinins and the lactoperoxidase–isothiocynate system. The agglutinins can induce clumping of starter-culture cells and slow

their growth and metabolism. The lactoperoxidase–isothiocynate system can inhibit starter cultures. Antimicrobials can cause problems only when raw milk is used, because both are destroyed by heating milk. Milk can also contain antibiotics, either used in the feed or used to treat animals for some infections, such as mastitis. Their presence can also affect the growth of starter cultures. Some milk can contain heat-stable proteases and lipases produced by some psychrotrophic bacteria, such as *Pseudomonas* species, during refrigerated storage of raw milk before pasteurization (see Chapter 21). These enzymes remain stable after heating and can cause product defects (low yield of cheese, proteolysis, rancidity). Before milk is used for fermentation, these aspects need to be considered.

B. Fermented Milk Products

Many types of fermented milk products are produced in different parts of the world. A few are produced by controlled fermentation, and the microbial types and their respective contributions are known. In many others, fermented either naturally or by back slopping, the microbial profiles and their contribution are not exactly known. Many types of lactic and bacteria and some yeasts are found to predominate microbial flora in these products,[3,4] some of which are listed:

1. *Buttermilk*. Made with *Lactococcus* species without or with *Leuconostoc cremoris*; some can have biovar diacetylactis in place of *Leu. cremoris* (such as ymer in Denmark), whereas some can have a ropy variant of *Lactococcus* species (langfil in Norway) or mold (*Geotrichum candidum* in villi in Finland).
2. *Yogurt*. Made with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; some types can also have added *Lab. acidophilus*, *casei*, *rhamnosus*, and *Bifidobacterium* spp.; some may also have *Lactococcus* species and *Lab. plantarum* and lactose-fermentating yeasts (dahi in India).
3. *Acidophilus Milk*. Made from *Lab. acidophilus*.
4. *Bifidus Milk*. Made from *Bifidobacterium* spp.
5. *Yakult*. Made from *Lab. casei*; may contain *Bifidobacterium* spp.
6. *Kefir.* Made from *Lab. kefir* (several species of yeasts along with *Leuconostoc, Lactobacillus*, and *Lactococcus* spp.).
7. *Kumiss.* Made from *Lab. delbrueckii* subsp. *bulgaricus* and yeasts.

Among these, cultured buttermilk and yogurt are discussed here.

C. Microbiology of Cultured Buttermilk Fermentation

It is produced from partially skim milk through controlled fermentation with starter cultures.

1. Product Characteristics

It should have a pleasant acid taste (from lactic acid) and high degree of aroma (from diacetyl), with slight effervescence (from CO_2). It should have white color with smooth, thick body and pour easily.

2. Processing

1. Skim milk ≥ 9% SNF + citrate (0.2%)
2. Heated at 185°F (85°C) for 30 min (kills bacterial cells and phages)
3. Cooled to 72°F (22°C), starter added, agitated for 50 min (incorporates air)
4. Incubated at 72°F (22°C) for 12 h, pH 4.7, acidity 0.9%
5. Gel broken, cooled to 40°F (4.5°C), and salted (package)

3. Starter (Controlled Fermentation)

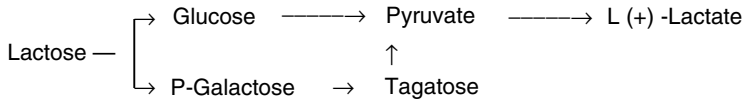
Lac. lactis ssp. *lactis* or *cremoris* is used for acid and *Leu. mesenteroides* ssp. *cremoris* for diacetyl and CO_2. They can be used as direct vat set frozen concentrates. (*Lac. lactis* ssp. *lactis* biovar diacetylactis is generally not used as it may produce too much acetaldehyde, causing green or yogurt flavor defect.)

4. Growth

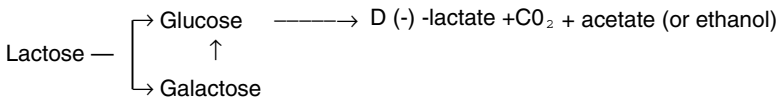
At 72°F, there is balanced growth of the two species, and balanced production of acid, diacetyl, and CO_2. Above 72°F, the growth of *Lactococcus* species is favored, with more acid and less flavor; below 72°F, the growth of *Leuconostoc* species is favored, with less acid and more flavor.

5. Biochemistry

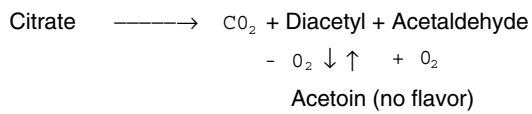
Lactose (transported by PEP-PTS system) is hydrolyzed by P-β-galactosidase in *Lactococcus* spp.:



Lactose hydrolysis by β-galactosidase of *Leuconostoc* sp.:



Citrate metabolism by *Leuconostoc* sp.:



For a desirable flavor, the diacetyl:acetaldehyde ratio should be >3:1 to <4.5:1.

6. Genetics

Lactococcus lactis strains should transport and hydrolyze lactose (Lac^+), metabolize P-galactose by the tagatose pathway and galactose by the Leloir pathway, be phage resistant, not produce slime, and not be very proteolytic.

Leuconostoc species should be able to transport and utilize citrate to produce more diacetyl and less acetaldehyde and ferment lactose, be phage resistant, and not produce slime.

Strains should not produce inhibitory compounds (such as bacteriocins) against each other, but can have antimicrobial activity toward undesirable organisms. Through selection and genetic manipulation, strains have been developed that grow rapidly, produce desirable characteristics, and are resistant to some phages. Current information on genome sequences of *Lactococcus* and *Leuconostoc* strains and their phages will help develop better strains in the future.

7. Microbial Problems

Because of too much acetaldehyde production (especially if biovar diacetylactis is used), green (yogurt flavor) may develop. A slimy texture implies contamination with bacteria that produce slime (*Alcaligenes faecalis*) or that starter cultures (some *Lac. lactic* strains) are slime formers (exopolysaccharides). A yeasty flavor implies contamination with lactose-fermentating yeasts, and a cheesy flavor alludes to contamination with proteolytic psychrotrophs (during storage). Proteolysis by proteases of contaminants in starters can also cause development of bitter flavor, especially during storage. (Also see chapter 19.)

D. Microbiology of Yogurt Fermentation

1. Characteristics

Plain yogurt has a semisolid mass due to coagulation of milk (skim, low, or full fat) by starter-culture bacteria. It has a sharp acid taste with a flavor similar to walnuts and a smooth mouth feel. The flavor is due to the combined effects of acetaldehyde, lactate, diacetyl, and acetate, but 90% of the flavor is due to acetaldehyde.

Many types of yogurt are available in the market, e.g., plain yogurt, fruit yogurt, flavored and colored yogurt, blended yogurt, sweetened yogurt, heated yogurt, frozen yogurt, dried yogurt, low-lactose yogurt and carbonated yogurt.[3,5]

2. Processing

Yogurt is generally fermented in batches, but a continuous method has also been developed. The batch process for a low-fat (2%) plain yogurt is as follows:

1. Homogenized milk (12% TS) + stabilizer (1%). The stabilizer is added to give desired gel structure.

2. Heated to 185°F (85°C) for 30 min, and cooled to 110°F (43.3°C). Heating helps destroy vegetative microbes and slightly destabilize casein for good gel formation.
3. Starter added, incubated at 110°F (29.5°C) to pH 4.8 for ca. 6 h, acidity ca. 0.9%. Starter used as either direct vat set (frozen) or bulk culture (2 to 3%).
4. Quickly cooled to 85°F in ca. 30 min to slow down further starter growth and acid production, especially by *Lactobacillus* species, agitated, and pumped to filler machine.
5. Packaged in containers, and cooled by forced air to 40°F (4.4°C). Final cooling by forced air results in a rapid drop in temperature to stop the growth of starters.
6. Held for 24 h; pH drops to 4.3.

3. Starters (Controlled Fermentation)

Frozen concentrates or direct vat set starters can be used. Normally, *Lab delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* are used. Some processors also combine these two with other species, such as *Lab. acidophilus* and *Bifidobacterium* spp., *Lab. rhamnosus,* or *Lab. casei*. However, in general, they do not compete well in growth with the two yogurt starters. Therefore, they are added in high numbers after fermentation and before packaging. They may not survive well when present in yogurt with the regular yogurt starter cultures.

For a good product, the two starter species should be added at a *Streptococcus:Lactobacillus* cell ratio of 1:1; in the final product, the ratio should not exceed 3:2. However, *Lactobacillus* cells are more susceptible to freezing and freeze drying. In a frozen concentrate starter for use as DVS, the survivors may not be present in a desired ratio unless they are properly preserved (see Chapter 13).

4. Growth

For balanced growth of the two species, the fermentation is conducted at ca. 110°F (43.3°C). At this temperature, both acid and flavor compounds are produced at the desired level. If the temperature is raised above 110°F, the *Lactobacillus* sp. predominates, causing more acid and less flavor production; at temperatures below 110°F, growth of *Streptococcus* sp. is favored, forming a product containing less acid and more flavor.

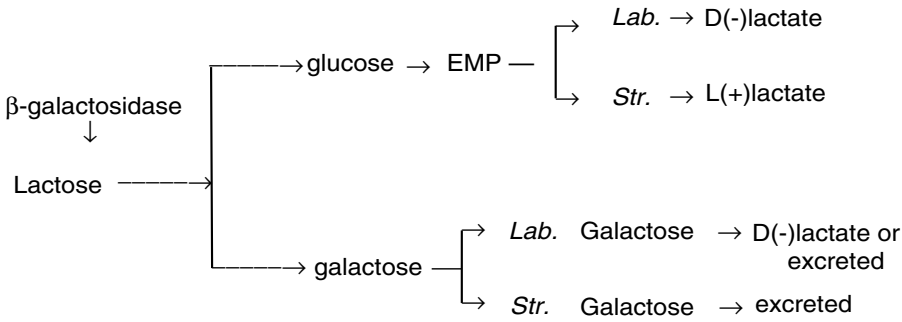
The two species show symbiotic growth while growing together in milk. Initially, *Streptococcus* sp. grows rapidly in the presence of dissolved oxygen and produces formic acid and CO_2. The anaerobic condition, formic acid, and CO_2 stimulate growth of *Lactobacillus* sp., which has good exoproteinase and peptidase systems and produces peptides and amino acids from milk proteins (outside the cells) in the milk. Some of the amino acids, such as glycine, valine, histidine, leucine, and methionine, are necessary for good growth of the *Streptococcus* sp., which lacks proteinase enzymes. *Streptococcus* sp. gets these from the milk and grows rapidly until the pH drops to ca. 5.5, at which time the growth of *Streptococcus* sp. slows down. However, growth of *Lactobacillus* sp. continues fairly rapidly until the temperature is reduced to 85°F, following a drop in pH to 4.8. At 85°F, both grow slowly, but *Streptococcus* sp. has the edge. At 40°F and a pH ca. 4.3, both species stop growing.

The two species also have a synergistic effect on growth rate, rate of acid production, and amounts of acetaldehyde formation when growing together as compared with when growing individually. The species growing separately in milk produce ca. 8 to 10 ppm acetaldehyde; when grown together, acetaldehyde production increases to a desirable level of 25 ppm or higher.

5. Biochemistry

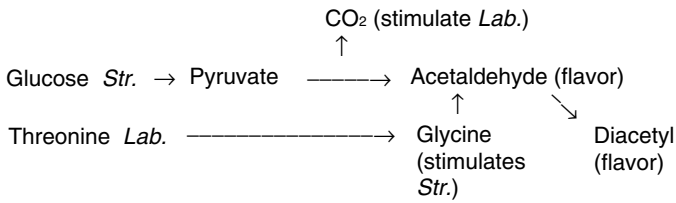
a. Lactose Metabolism

Both species have a constitutive β-galactosidase system, and lactose (transported by permease systems) is hydrolyzed to glucose and galactose. Both species are homofermentative and produce lactate from glucose by the EMP pathway. *Lab. delbrueckii* ssp. *bulgaricus* strains have enzymes for the Leloir pathway to metabolize galactose, but while actively metabolizing glucose they do not utilize galactose well. Most *Str. thermophilus* strains do not have the enzymes of the Leloir pathway (or have a very weak system) and thus do not metabolize galactose. As a result, galactose is excreted outside, causing its accumulation in yogurt.



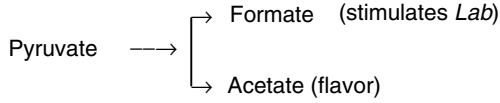
b. Flavor Production

The major flavor compound in yogurt is acetaldehyde (25 ppm), with some diacetyl (0.5 ppm) and acetate. Acetaldehyde is produced in two ways: from glucose via pyruvate by *Streptococcus* sp. and from threonine (supplied or produced through proteolysis in milk) by *Lactobacillus* sp.



c. Formate Production

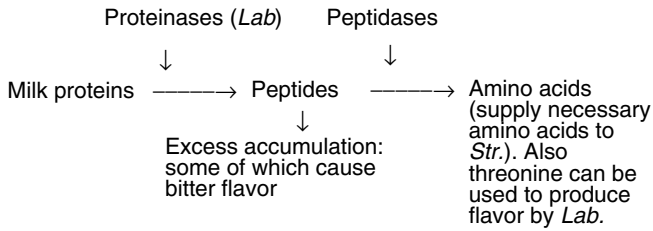
Formate (necessary for *Lactobacillus* growth) is produced by *Str. thermophilus* from pyruvate by the action of formate lyase.



d. Slime Formation (Glycan)

β-galactosidase in some strains of *Str. thermophilus* polymerizes glucose to produce oligosaccharides and glycan, which may give a viscous texture to yogurt.

e. Proteolysis


Proteinases (Lab)
Peptidases
Milk proteins
Peptides
Amino acids (supply necessary amino acids to Str.). Also threonine can be used to produce flavor by Lab.
Excess accumulation: some of which cause bitter flavor

6. Genetics

- *Lac⁺ Phenotype.* In both species, the trait (β-galactosidase and permease) is chromosomally linked, constitutive, and quite stable. Some strains can have strong β-galactosidase activity.
- *Gal⁺ Phenotype. Lab. delbrueckii* ssp. *bulgaricus* is Gal^+, but *Str. thermophilus* usually is a Gal^- phenotype. Strains with a good Gal^+ phenotype can be developed to reduce galactose accumulation in the product.
- *Pro⁺ Phenotype*. *Lab. delbrueckii ssp. bulgaricus* strains differ in protein hydrolysis ability. Strains with desirable proteolytic activity should be used. Too much proteolysis can adversely affect the texture and enhance development of bitter flavor.
- *Phage Resistance.* Both species have phages; resistant strains need to be developed and used.
- *Symbiotic and Synergistic Relationship*. Strain selection for best combinations is necessary.
- *Antagonistic Effect.* Strains should not produce inhibitory compounds (such as bacteriocins) against each other.
- *Good Survival to Freezing and Drying*. Possible genetic basis needs to be studied to develop resistant strains to produce concentrated starter cultures.

Complete genome sequences of several strains of each species are available now, which will help in the future to develop more desirable strains for use in yogurt production.

7. Microbial Problems

In plain yogurt, flavor problems can be associated with the concentrations of acetaldehyde. A low concentration gives a chalky and sour flavor, and too much acetaldehyde can give a green flavor. Similarly, too much diacetyl gives a buttery aroma. Too much acid production during storage causes a sour taste. Proteolysis and accumulation of bitter peptides during storage are associated with bitter flavor. Production of exopolysaccharides by the starter can give a viscous and ropy texture (which can be desirable in some situations). Growth of yeasts during storage can also produce a fruity flavor, especially in yogurt containing fruits and nuts. In colored, flavored, and blended yogurt, many of these problems are masked. During long storage, molds can grow on the surface (also see Chapter 19).

E. Cheeses

Cheeses are made by coagulating the casein in milk with lactic acid produced by lactic acid bacteria and without or with the enzyme rennin, followed by collecting the casein for further processing, which may include ripening.[6] The process was probably accidentally discovered in the Middle East ca. 7000 B.C. from the coagulation of milk stored in a calf stomach by lactic acid produced by lactic acid bacteria (and probably rennin in the stomach). At present, many varieties of cheeses are made worldwide, which probably use more than 20% of the total milk produced. In the U.S., the total production of different varieties of cheese in 1982 was 4.4 billion pounds (2 billion Kg) and, in 1987, increased to 5.3 billion pounds (2.4 billion Kg). Because of the worldwide increase in cheese consumption, cheese production will continue to increase not only in the U.S., but also in other countries, especially in dairy-rich countries in Europe and in New Zealand.

Cheese varieties have been grouped in different ways. Examples of several varieties based on starter cultures used and some important secondary flora are listed here.

1. Unripened Cheese

- Soft
  Cottage cheese with starters *Lac. lactis* ssp. *lactis* and *cremoris* and *Leuconostoc mesenteroides* ssp. *cremoris*.
  Mozzarella cheese with starters *Str. thermophilus* and *Lab. delbrueckii* ssp. *bulgaricus*.

2. Ripened Cheese

- Soft
  Brie cheese with starter *Lac. lactis* ssp.; *Penicillium* sp. and yeasts are secondary flora.

- Semihard
  Gouda cheese with starters *Lac. lactis* ssp. and *Leuconostoc* spp.; dairy *Propionibacterium* may be secondary flora.
  Blue cheese with starter *Lac. lactis* ssp., *Leuconostoc* spp.; *Penicillium roquefortii*, yeasts, and micrococci are secondary flora.
- Hard
  Cheddar cheese with starters *Lac. lactis* ssp.; some lactobacilli and pediococci (and probably enterococci) are secondary (or associative) flora.
  Swiss cheese with starters *Str. thermophilus, Lab. helveticus*, and dairy *Propionibacterium* spp.; enterococci can be secondary (associative) flora.

Only cottage, cheddar, Swiss, and blue cheeses are further discussed to understand the microbiological aspects of unripened and ripened cheeses.

F. Microbiology of Cottage Cheese[6]

1. Characteristics

Cottage cheese is made from low-fat or skim milk and has a soft texture with ca. 80% moisture. It is unripened and has a buttery aroma due to diacetyl (along with lactic acid and little acetaldehyde).

2. Processing (from Skim Milk)

1. Pasteurized, cooled to 70°F (22.2°C) , starter added, and incubated for 12 h at pH 4.7.
2. Firm curd set, cut in cubes, and cooked at 125°F (51.7°C) for 50 min or more.
3. Whey drained off, stirred to remove more to get dry curd.
4. Salted, creamed, and preservative added.
5. Packaged and refrigerated.

3. Starters (Controlled Fermentation)

Frozen concentrate for direct vat set can be used. Mixed strains of *Lac. lactis* ssp. *cremoris* and *lactis* are predominantly used for acid. *Leu. mesenteroides* ssp. *cremoris* can be added initially (in which case citrate is added to milk), mainly for diacetyl. *Lac. lactis* ssp. *lactis* biovar diacetylactis can be used for diacetyl, but not inoculated in milk because of formation of too much CO_2, which causes curd particles to float. Instead, it is grown separately in cream, which is then used to cream the dry curd.

4. Growth, Biochemistry, and Genetics

Growth, biochemistry, and genetics (including current strain improvements) are similar to those described for the microbiology of buttermilk (see Section IIIC).

5. Microbial Problems

- *Slow Growth*. Weak or loss of Lac^+ phenotype, phage attack or less viable cells in frozen concentrate, antagonistic effect among starter strains for reasons described previously.
- *Floatation of Curd.* Caused by too much CO_2 production by flavor-producing starters.
- *Harsh Flavor.* Caused by more acetaldehyde and less diacetyl production from metabolic imbalance and reduced environment.
- *Low Yield.* Caused by partial proteolysis of casein by heat-stable proteinases produced in refrigerated raw milk by psychrotrophs such as *Pseudomonas* spp.
- *Flavor Loss.* Caused by reduction of diacetyl to acetoin in a reduced environment as well as by growth of undesirable bacteria during storage, such as *Pseudomonas* spp.
- *Spoilage.* Because of high moisture and low acid content, spoilage psychrotrophic bacteria (such as *Pseudomonas* spp.), yeasts, and molds can grow during storage. Preservatives such as sorbates can be used to extend shelf life under refrigerated storage (also see Chapter 19).

G. Microbiology of Cheddar Cheese[6]

1. Characteristics

Cheddar cheese is made from whole milk, contains less than 39% moisture, 48% fat, is generally orange-yellow in color (due to added color), and ripened. Smoothness of texture and intensity of characteristic flavor vary with the starters used and the period of ripening. The typical flavor is the result of a delicate balance among flavor components produced during ripening through enzymatic breakdown of carbohydrates, proteins, and lipids in unripened cheddar cheese.

2. Processing

1. Pasteurized; color (annatto) and starter added.
2. Incubated at 86°F (30°C) for acidity to increase by 0.2%; rennet added.
3. Incubated for coagulation (ca. 30 min) and cut in cubes.
4. Cooked at 100°F (37.8°C), whey drained, cheddaring to lose whey and curd to mat.
5. Milled, salted, put in form, pressed for 16 h to drain whey, and removed.
6. Dried for 5 d at 50°F (10°C), waxed, and cured at 40°F (4.4°C) for 2 to 12 months.

3. Starters (Controlled Fermentation)

Starters include selected mixed strains of *Lac. lactis* ssp. *cremoris* or *lactis*. *Leuconostoc* may be added for flavor. Starters can be used as frozen concentrates (direct vat set).

4. Growth

Growth is accomplished by incubating at 86°F (30°C) for mesophilic starters to start early growth and produce lactic acid to a 0.2% level by 60 min. This is important for the coagulation of casein to occur rapidly following the addition of rennin. During curing, cells of the starter (also of secondary flora) slowly die and release intracellular enzymes in the cheese.

5. Biochemistry

A large number of biochemical reactions occur during the different stages of processing, from the initial incubation to the end of ripening, many not yet properly understood. Some are described here.

Initially, lactose metabolism produces lactic acid as well as some diacetyl, acetate, ethanol, and acetaldehyde, especially under aerobic conditions. Rennin destabilizes casein to produce paracasein, which coagulates at low acidity and low temperature. In addition, extracellular proteinases and peptidases of the starter cultures metabolize milk proteins to peptides and amino acids and transport them into the cells. Normally very little, if any, lipolysis occurs due to microbial growth.

During curing, breakdown of remaining lactose in the curd continues. However, a large change occurs in proteins and other nitrogenous compounds. By the action of rennin (retained in curd) and cellular exo- and endoproteinases and peptidases, peptides of different sizes and amino acids are released. Further breakdown of amino acids produces hydrogen sulfide, methanethiol and related sulfur compounds, amines, and other products. Lipids also undergo lipolysis, releasing fatty acids, including the C_4 to C_8 fatty acids (which are present in milk fat). Other reactions produce lactones, ketones, and thioesters. Some of the reactions are nonenzymatic. The typical Cheddar cheese flavor is the result of a delicate balance among the products produced from carbohydrate, protein, and lipid breakdown during processing and curing. The concentrations of these components change with curing time.

Some secondary microflora that survive heating or gain entrance later in the milk and curd during processing have definite roles in the flavor of cheddar cheese. These include some enterococci, lactobacilli, pediococci, micrococci, and some Gram-negative rods. They probably contribute to the typical intense flavor that could be missing in cheese made with defined starter strains only. Some of these flora are known to produce several flavor compounds rather rapidly and at higher concentrations (e.g., volatile fatty acids and H_2S).

6. Genetics

Phenotypic characteristics, as described before for these species, should be considered. Lac^+ strains capable of producing lactic acid rapidly at the initial stage are preferred. Also, strains with weak proteinases (Pro^+) activity are desirable because they do not cause rapid proteolysis with the accumulation of some peptides and the appearance of bitter flavor in the products. In mixed starters, they should not have an antagonistic effect. Also, the strains should preferably be resistant to multiple phages.

7. Microbial Problems

Bitter flavor in cheddar cheese, especially in the aged product, results from the accumulation of bitter peptides that are ca. 1000 to 12,000 Da and rich in hydrophobic amino acids. Starters capable of hydrolyzing proteins rapidly (fast starters) tend to produce bitter peptides more than slow starters do. Their enzymes hydrolyze proteins quickly, releasing large amounts of peptides that are subsequently hydrolyzed slowly to smaller peptides and amino acid by peptidases, resulting in the accumulation of peptides. Because they are hydrophobic, bitter peptides are generally hydrolyzed slowly, causing them to accumulate. Use of slow starters for protein breakdown at a slow rate or treatment of cheese with peptidase, or both, are effective in reducing bitterness (also see Chapter 19).

Mold growth on the surface or in air pockets inside the cheese can occur after removing the packing material from cheese. The spores are generally present in the raw material or get in the product during processing and before sealing. It is not possible to determine from the colonial morphology whether they are mycotoxin producers. It is better not to consume cheese with heavy growth.

Staphylococcus aureus, following contamination of milk after heating, can grow during processing cheddar cheese and produce enterotoxins. The toxins remain in the cheese even after the death of cells during curing. Food poisoning can occur from consuming such cheese (even if they are heated in some preparations).

Biological amines (histamine from histidine, and tyramine from tyrosine) can form by decarboxylation (by starter decarboxylases) of some amino acids, especially in cheese ripened for a long time. Allergic reactions can occur from consuming such cheese. Secondary flora may have an important role in such amine formation.

H. Microbiology of Swiss Cheese[6]

1. Characteristics

Swiss cheese is made from partially skimmed milk (cow's) and coagulated with acid and rennin; it is hard and contains ca. 41% moisture and 43% fat. The cheese should have uniformly distributed medium-sized eyes (openings). It has a sweet taste, due to proline, and a nutty flavor.

2. Processing

1. Pasteurized, starter added, and incubated at 90°F (32.2°C) for acidity to increase by 0.2%.
2. Rennin added, incubated for firm coagulation, cut in 1/8-in. cubes, and cooked for 1 h at 125°F (51.7°C).
3. Whey removed, curd pressed for 16 h, cut in blocks, and exposed in brine (55°F) (12.8°C) for 1 to 3 d.
4. Surface dried, vacuum packaged, stored for 7 d at 55°F (12.8°C), and transferred to 75°F (23.9°C) for 1 to 4 weeks.
5. Cured at 37°F (2.8°C) for 3 to 9 months.

3. Starters (Controlled Fermentation)

Starters are *Str. thermophilus* and *Lab. helveticus* as primary for acid and *Propionibacterium* sp. as secondary for eye formation, taste, and flavor.

4. Growth

Primary starters grow during fermentation, cooking, and processing; growth of *Str. thermophilus* is favored. Propionibacteria grow well during storage at 75°F (23.9°C). During curing, none of the starters grow. The cells slowly die and release intracellular enzymes.

5. Biochemistry

Lactose is hydrolyzed by β-galactosidase of both lactic acid bacteria and metabolized by the EMP pathway. *Str. thermophilus* produces L(+)-lactic acid and *Lab. helveticus* produces D(–)-lactic acid. Some acetate also forms. Production of large amounts of lactate is very important to facilitate growth of propionibacteria. During storage at 75°F (23.9°C), propionibacteria convert lactate (by lactate dehydrogenase) to pyruvate, which is then converted to propionic acid, acetate, and CO_2. Eye formation occurs from the production of CO_2, and the size and distribution of eyes depend on the rate of CO_2 production. Proteins are hydrolyzed by rennin, intracellular proteinases, and peptidases of starters, especially propionibacteria, resulting in the production of small peptides and amino acids, which impart the nutty flavor and sweet taste (sweet taste is attributed to a relatively high concentration of proline produced by propionibacteria). Very little lipolysis occurs during curing.

6. Genetics

Rapid production of lactate in large amounts from lactose by lactic acid bacteria and their resistance to phages (some *Lab. helveticus* strains have temperate phages that get activated at high processing temperature, causing starter failure) are important considerations. *Propionibacterium* strains should produce CO_2 at proper rates for desirable numbers and sizes of eye formation (uniform distribution of medium-size eyes preferred).

7. Microbial Problems

Spores of *Clostridium tyrobutyricum*, present in raw milk or entering as contaminants, can germinate, grow, and cause rancidity and gas blowing in this low-acid cheese. Nisin has been used as a biopreservative to control this problem (also see Chapter 19).

I. Microbiology of Blue Cheese[6]

1. Characteristics

Blue cheese is a semihard (46% moisture, 50% fat), mold-ripened cheese made from whole milk (cow's). It has a crumbly body, mottled blue color, and sharp lipolytic flavor.

2. Processing

1. Homogenized, pasteurized, starter added, and incubated at 90°F (32.2°C) for acidity to increase to 0.2%.
2. Rennet added, incubated for firm set, cut, cooked at 100°F (37.8°C), and whey drained.
3. Curd collected in hoop, drained 16 h, and salted in brine for 7 d.
4. Spiked to let air get inside. Mold spores added, stored at 50°F (10°C) in high humidity for 4 weeks.
5. Stored at 40°F (4.4°C) for curing for 3 months.

3. Starters and Growth (Controlled Fermentation)

Lac. lactis ssp. *cremoris* or *lactis* and *Leu. cremoris* or *lactis* serve as primary starters. *Pen. roquefortii* spores serve as secondary starters. The lactic starters grow until curing, and from lactose, they produce lactate, diacetyl, acetate, CO_2, and acetaldehyde. Mold spores, during storage at 50°F (10°C) in high humidity, germinate quickly, produce mycelia, and spread inside to give the mottled green appearance. Their growth continues during curing. Puncturing the inside of the cheese helps remove CO_2 and lets air in to help the growth of molds.

4. Biochemistry, Genetics, and Problems

Lactococcus species produce mainly lactic acid from lactose, whereas *Leuconostoc* species produce lactic acid, diacetyl, CO_2, and acetate. Proteolysis is quite limited by the lactic starters. Molds produce extracellular lipases and proteinases and cause lipolysis and proteolysis during curing. Fatty acids are both oxidized and reduced to produce methyl ketone and δ-lactone, respectively. These, along with volatile fatty acids, contribute to the sharp flavor of blue cheese.

The desired genotypes of the lactic acid bacteria starters used have been previously described. A white variant of the mold has been isolated and used to produce this cheese without the blue mottled color. *Pen. roquefortii* strains that produce mycotoxins have been identified. Strains that do not produce mycotoxins need to be selected. Ripening for a long time can lead to formation of biologically active amines from some amino acids (e.g., histamine from histidine).

J. Accelerated Cheese Ripening[7]

During curing of hard and semihard cheeses, milk components are degraded through enzymatic (from starters and secondary flora) and nonenzymatic reactions. This is necessary to develop a desirable flavor of these cheeses. However, because of a long storage time, it is not quite economical. Thus, methods are being studied that will speed up the ripening process. Some of these methods are briefly described.

1. Curing at High Temperature

Because enzymatic (also nonenzymatic) reactions increase as the temperature is increased, studies were performed to cure cheese above the usual 5 to 6°C (40°F).

Ripening some cheeses at 13 to 16°C (55 to 61°F) reduced the curing time by 50% or more. However, at higher temperature, growth of spoilage bacteria has been a problem in some cheeses, and there is some concern about the growth of foodborne pathogens.

2. Addition of Enzymes

As intracellular enzymes of starters have an important role in curing, enzymes obtained from cell lysates of starter-culture bacteria have been added to increase the rate of curing. In cheddar cheese, the curing time has been substantially reduced, but has resulted in bitter flavor.

3. Slurry Method

Cheddar cheese slurry has been prepared by mixing water with cheese to 40% solids (in place of the usual 60%). The slurry is incubated at 30°C for 4 to 5 d with agitation. This method increases the flavor greatly and the product can be used to make processed cheeses. The major disadvantages are the inability to properly control the enzymatic reactions to produce uniform products and possible growth of spoilage and pathogenic bacteria.

4. Novel Methods

In recent years, application of a suitable bacteriocin (of lactic acid bacteria) to cheese or exposing cheese to high hydrostatic pressure to lyse the cells of starter cultures is being studied to enhance ripening by the released intracellular enzymes.

IV. FERMENTED MEAT PRODUCTS

A. Types

Fermented meat products are produced by first mixing meat, fat, salt, sugar, curing agents, and spices; filling the mixture in a casing; and fermenting it either naturally or by adding (during mixing) selected starter-culture bacteria.[3] The acids produced by the starters during fermentation and the curing agents used help control the growth of pathogenic and spoilage bacteria that might be present in the meat. Depending on the type, the fermented products may be dried to reduce A_w or smoked or heated to ensure the safety and shelf life of the products.

Meat fermentation probably originated in the Mediterranean countries and later spread to European countries and North America. In the U.S., semidry sausages are most popular, although some dry sausages are also produced. Following fermentation, semidry sausages are heated (also sometimes smoked) before consumption. For dry sausages, following cooking, the products are dried to reduce the A_w. Even now, fairly large amounts of fermented sausages in the U.S. are produced by natural fermentation, especially those produced by small processors. However, more pro-

cessors now use selected starter cultures and controlled fermentation. Commercial starter cultures are available as both frozen and freeze-dried concentrates for direct inoculation in the meat mixture.

Semidry and dry sausages include many types, such as pepperoni, Genoa salami, hard salami, summer sausage, beef sticks, beef logs, thuringer, cervelat, and Italian salami. Most are made with beef and pork, but in recent years, some have been made with meat from chicken and turkey. The microbiology of semidry sausages is described here.

B. Microbiology of Semidry Sausages

1. Characteristics

Semidry sausages include summer sausage, thuringer, and semidry salami. The average composition is ca. 30% fat, 20% protein, 3% minerals (salts), and 47% water. They have a tangy taste with a desirable flavor imparted by the combined effect of lactate, acetate, and diacetyl, and some breakdown components from proteolysis and lipolysis. The use of spices also contributes to the flavor. Those containing nitrite have a pinkish color in contrast to the grayish color in products without it.

2. Processing

1. Meat, salts, glucose, cure, spices, and starter mixed uniformly.
2. Stuffed in casings, fermented at 85 to 110°F (29.4 to 43.3°C) with 80 to 90% relative humidity.
3. Incubated until the pH drops to ca. 5.2 to 4.6, cooked to 140°F (60°C) internal temperature, and cooled to 50°F.
4. Stored at 40 to 50°F (4.4 to 10°C) for 3 to 4 d, vacuum-packaged, and consumed directly.

Cures contain nitrite to give a final concentration of ca. 100 ppm. Fermentation can be carried out in a smokehouse. Fermentation time is usually 8 to 12 h, during which the pH is dropped to desired level.

3. Starters (Controlled or Natural Fermentation)

In controlled fermentation, frozen or dried concentrates are used directly at 10^{6-7} cells/g mix. Starters should not be mixed with salt, cure, or spices as it can kill injured cells. Instead, they should be thawed and immediately put into the meat. Starters vary, depending on the fermentation temperature and final pH of the product desired. For high temperature and low pH, *Pediococcus acidilactici* strains are preferred; for low temperature and high pH, *Lab. plantarum* strains are preferred. *Ped. pentosaceus* strains can be used under both conditions. Some starters can have both *Pediococcus* and *Lactobacillus* species. In addition, selected *Micrococcus* spp. or *Sta. carnosus* strains are added as secondary flora for their beneficial effects on desired product color.

In naturally fermented sausages, *Lab. sake, Lab. curvatus,* and *Leuconostoc* spp. present in raw materials are important starter bacteria, especially when fermentation is set at lower temperatures (60 to 70°F [15.6 to 21.1°C]) for several days and the final pH reached is not below 5.0.

4. Growth

Because the raw meat used may contain pathogens and spoilage bacteria, it is extremely important that starter culture grows rapidly and produces acid in large amounts to reduce pH from the initial 5.7 to ca. 5.3 very quickly to retard their growth. This can be achieved by adding large numbers of active starter cells, adding dextrose to the mix, and setting the temperature of fermentation optimum for the starters used. The optimum growth temperatures for *Ped. acidilactici, Ped. pentosaceus*, and *Lab. plantarum* are ca. 40, 35, and 30°C (104, 95, 86°F), respectively. *Micrococcus* spp. and *Sta. carnosus* grow well at ca. 32.2°C (90°F). Cooking to an internal temperature of 60°C (140°F) kills *Lab. plantarum* and probably *Ped. pentosaceus*, but probably not *Ped. acidilactici*, *Micrococcus*, or *Sta. carnosus*. However, low pH and low A_w prevent their growth in the finished products.

5. Biochemistry

Both pediococci are homolactic fermentors and metabolize glucose to mainly lactic acid (DL), with small amounts of acetate and diacetyl. *Lab. plantarum*, being facultatively heterofermentative, metabolizes glucose to principally lactic acid (DL); however, it can also produce substantial amounts of acetate, ethanol, and diacetyl. Strains of all three species can produce H_2O_2, which can discolor the product by oxidizing myoglobin during fermentation. *Micrococcus* spp. or *Sta. carnosus* have catalase that can destroy H_2O_2. *Micrococcus* sp. or *Sta. carnosus* and some strains of *Lab. plantarum* can also reduce nitrate to nitrite. If nitrate is used in place of nitrite in cure, these bacteria can produce nitrite and help develop the agreeable pinkish color of the product. If the products are cured or stored for long periods of time, some of the intracellular enzymes of the lysed cells of starters are able to cause proteolysis and lipolysis and produce biologically active amines (such as histamine).

6. Genetics

Rapid-acid-producing lactic acid bacterial strains at temperatures of fermentation and non-H_2O_2 producers are desired. Strain selection can also be done for nonproducers of biogenic amines. Strains producing bacteriocins can be used to control pathogens and spoilage bacteria. *Ped. acidilactici* strains that cannot hydrolyze sucrose (Suc^-) can be used to produce sweet and sour products by supplementing the meat mixture with both glucose and sucrose. Exopolysaccharides-producing strains can improve the texture of products.

7. Microbial Problems

Slow acid production can be a serious problem if the starters used are not metabolically active or have lower numbers of viable cells or due to other factors such as low glucose and high salts in the mix. Sour or no flavor can occur if the starter, especially *Ped. acidilactici*, grows very rapidly and reduces the pH to below 4.5. Gas formation can occur because of growth of *Leuconostoc* spp. during fermentation and during storage in vacuum packages. *Leuconostoc* spp. are usually present in raw meat. Pathogens, when present in meat, can grow if acid production is slow during fermentation. Acid-resistant pathogens can also survive in the products and cause health hazards. During long storage or curing, biogenic amines can form. Also, mycotoxin-producing molds can grow on the product surface during curing (also see Chapter 19).

V. FERMENTED VEGETABLE PRODUCTS

Almost all vegetables can be fermented through natural processes, because they harbor many types of lactic acid bacteria. Worldwide, many types of vegetables are fermented, mostly in small volumes. However, some are produced commercially. Vegetable fermentation originated in the early years of human civilization and even now is widely used by many cultures. Examples of some fermented products and vegetables used currently for fermentation are sauerkraut (from cabbage), olives, cucumbers, carrots, celery, beans, peas, corn, okra, tomatoes, cauliflower, peppers, onions, citron, beets, turnips, radishes, chard, Brussels sprouts, and their blends. Most are produced by natural fermentation; however, some, such as cucumbers, are currently produced in limited amounts by controlled fermentation. Production of sauerkraut by natural fermentation is described here as an example.

A. Microbiology of Sauerkraut[3]

1. Characteristics

Sauerkraut is produced by fermenting shredded cabbage. The product has a sour taste with a clean acid flavor.

2. Processing

1. Cabbage cleaned, trimmed, and shredded fine and uniform.
2. Packaged tight to exclude air in vat, and layered with salt (2.25%).
3. Top covered to exclude air, and fermented at 18°C (65°F) for 2 months.

Fine shredding helps the sugars (3 to 6%) come out of cabbage cells. Tight packaging helps create an anaerobic condition, thus preventing the growth of aerobes. Salt stimulates growth of some lactic acid bacteria, and discourages the growth of some undesirable bacteria and pectinase (in cabbage) action. The top is covered to exclude air and prevent growth of some aerobes. Fermentation at 18°C (65°F) discourages the

rapid growth of some undesirable bacteria (facultative anaerobic or anaerobic), but encourages the growth of desirable lactic acid bacteria. Natural inhibitors in cabbage also discourage the growth of undesirable Gram-negative and Gram-positive bacteria.

3. Starters (Natural) and Growth

The raw material has a large number of undesirable organisms and a small population of lactic acid bacteria (<1%). Among the lactic acid bacteria, most are *Lactococcus* spp. and *Leuconostoc* spp., and a small fraction is *Lactobacillus* spp. and *Pediococcus* spp. During fermentation, sequential growth of these lactic acid bacteria occurs. The presence of 2.25% salt, large amounts of fermentable sugars (sucrose, hexoses, pentoses), absence of oxygen, and low fermentation temperature facilitate *Leuconostoc* spp., primarily *Leu. mesenteroides*, to grow rapidly. When the acidity has reached to ca. 1% (as lactic acid), growth of *Leu. mesenteroides* slows down. Then *Lab. brevis* starts growing rapidly until acid production reaches ca. 1.5%. Then *Ped. pentosaceus* takes over and increases the acidity to ca. 1.8%. Finally, *Lab. plantarum* starts growing and brings the acid level to ca. 2%.

4. Biochemistry

Leuconostoc spp. metabolize sucrose, hexoses, and some pentoses in the raw material to lactate, acetate, ethanol, CO_2, and diacetyl. *Lab. brevis* (obligatory heterofermentative, such as *Leuconostoc* spp.) ferments sucrose, hexoses, and pentoses to products similar to those by *Leuconostoc* spp. *Ped. pentosaceus* metabolizes hexoses to form mainly lactic acid and some pentoses to lactic acid, acetate, and ethanol. *Lab. plantarum* also produces products from sucrose, hexoses, and pentoses similar to those by *Ped. pentosaceus*. *Leuconostoc* spp. produces D(–)-lactate, whereas the other three species produce DL-lactate.

The characteristic flavor of sauerkraut is the result of the combined effects of lactate, acetate, ethanol, CO_2, and diacetyl in proper amounts.

5. Genetics

If starters are developed in the future for controlled fermentation, some of the characteristics that will be important are rapid acid production, good flavor production, low CO_2 production (to reduce gassy defect), and the ability to produce antimicrobial compounds, especially against pathogens.

6. Microbial Problems

Off-flavor, soft texture, and discoloration of sauerkraut can occur by growth of molds and yeasts when air is not completely excluded. A slimy texture of sauerkraut can occur due to overgrowth of *Leuconostoc* spp. in the presence of sucrose; they metabolize fructose but synthesize dextrans from glucose (also see Chapter 19).

VI. CONCLUSION

Food fermentation involves use of specific starter cultures in different types of foods as starting materials. The starting materials can be milk, meat, fish, fruits, vegetables, grains, seeds, and others. They are used separately or in combination. Current trend is to use controlled fermentation in place of natural fermentation. Improvement of strains has helped overcome some of the drawbacks of controlled fermentation. Information on genomes will help improve strains in the future.

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QUESTIONS

1. Define and discuss the advantages of different methods used to produce fermented foods.
2. Briefly discuss the factors to be considered while selecting milk to produce fermented dairy products.
3. List the characteristics of buttermilk and describe how they help select specific starter cultures.
4. Describe the symbiotic growth of starter cultures used in the production of yogurt. What genetic improvement will be important in these strains?
5. Most commercial yogurt now contains *Lab. acidophilus* and *Bifidobacterium* with regular yogurt bacteria. However, they are added to yogurt after fermentation. Briefly discuss the reasons.
6. In creamed cottage cheese, cream ripened with diacetyl-producing lactic starter is often used instead of using this starter during fermentation. Discuss the reasons.
7. Discuss the biochemical basis of the typical flavor of cheddar cheese. What is the basis of bitter flavor formation? How can it be reduced by genetic improvement of the starters?
8. Discuss the role of starter cultures in the development of desired characteristics in Swiss cheese.
9. Describe the role of molds in blue cheese production. What precautions should be taken in selecting the mold strains?

10. Discuss the methods used to accelerate cheese ripening.
11. List the primary and secondary starter cultures used in controlled fermentation of semidry sausages and discuss their specific roles.
12. Describe the sequential growth of lactic acid bacteria during the natural fermentation of sauerkraut.

CHAPTER 15

Intestinal Beneficial Bacteria

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I. INTRODUCTION

Since the discovery of food fermentation, our ancestors recognized that the process yielded products that had not only better shelf life and desirable qualities but also some health benefits, especially to combat some intestinal ailments. The belief in the health benefits of fermented foods continued throughout civilization and even today remains of interest among many consumers and researchers.[1] There are differences between early beliefs and the current interest: whereas the early beliefs probably emerged from associated effects (benefit from consuming) without knowing the scientific basis, current interest is based on understanding the microbiology and biochemistry of fermented foods, microbial ecology of the human gastrointestinal (GI) tract, and roles of some bacteria in the GI tract and in fermented foods in human health. Researchers, however, are divided in their opinions. Some advocate different health benefits that consumers can have from consuming fermented foods, especially fermented dairy products, but others doubt those attributes, especially when "cure-all" claims are made by overzealous individuals with none or very little scientific background in the area.

In Western countries, the beneficial role of fermented milk (yogurt) in the prolongation of life was first advocated by Metchnikoff in 1907. He suggested that the bacteria and their metabolites in yogurt neutralized the harmful products yielded from foods in the GI tract and provided protection to human health. However, later studies produced controversial results.

In recent years, especially since the 1970s, consumers in many developed countries have been particularly interested in the health benefits of foods. There is an increased interest in foods that are not harshly processed and preserved, but are natural, and fermented foods are considered natural and healthy. Consumers' interest in and demand for fermented foods have resulted in a large production increase of many products that had very small markets before. Many ethnic products have earned commercial status. In addition, consumers' interest in the health benefits of some bacterial species has stimulated production of new products containing live cells of these bacteria. These products are generally designated as probiotics. The term has been used loosely for a while, but is currently defined as a product containing living microorganisms, which on ingestions in certain numbers exert health benefits beyond inherent general nutrition.[2]

This trend will continue not only in the developed countries but also in many developing countries, particularly where fermented foods have been consumed for a long period of time. However, it will be extremely important to resolve the present controversies on the health benefits of probiotics, which can only be possible by understanding the current problems, conducting well-designed experiments, and

analysing the results scientifically. In this chapter, the current status of our knowledge on intestinal microbial ecology, beneficial microorganisms of the GI tract, beneficial effects of fermented milk products, and the probable causes of some controversies are briefly presented.

II. MICROBIOLOGY OF THE HUMAN GI TRACT

The GI tract of humans contains more than 10^{14} microorganisms, many more than the total number of our body cells. They are metabolically diverse and active; thus it is quite likely that they have a great influence on our well-being. It is estimated that the human GI tract harbors ca. 1000 bacterial species, but only 30 to 40 species constitute 95% of the population.[2] Normally, the microbial level in the small intestine (particularly in the jejunum and ilium) is ca. 10^{6-7}/g, and in the large intestine (colon) ca. 10^{9-10}/g of the content. The most predominant types in the small intestine are several species of *Lactobacillus and Enterococcus*, and in the large intestine are several genera of *Enterobacteriaceae*, different species of *Bacteroides, Fusobacterium, Clostridium, Eubacterium, Enterococcus, Bifidobacterium*, and *Lactobacillus*.[3]

The intestine of a fetus in the uterus is sterile. At birth, it is inoculated with vaginal and fecal flora from the mother. Subsequently, a large variety of microorganisms enter in the digestive tract of infants from the environment. From these, the normal flora of the GI tract are established. In both breast-fed and formula-fed babies during the first couple of days, *Escherichia coli* and *Enterococcus* appear in large numbers in the feces. Then, in the breast-fed babies, large numbers of *Bifidobacterium* species and a lower level of both *Esc. coli* and *Enterococcus* species appear. In formula-fed babies, in contrast, *Esc. coli* and *Enterococcus*, together with *Clostridium* and *Bacteroides*, predominate, with *Bifidobacterium* being almost absent; this situation may lead to diarrhea. As breast-fed babies are introduced to other foods, the levels of *Esc. coli, Enterococcus, Bacteroides, Clostridium*, and others increase, but *Bifidobacterium* still remains high. When breast-feeding is completely stopped, *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* species predominate, along with some *Esc. coli, Enterococcus, Clostridium*, and others. By the second year of life, the different microflora establish themselves at their specific ecological niche in the GI tract, and the population resembles that of adult GI tracts.

The intestinal microflora are divided into indigenous (autochthonous) and transient (allochthonous) types. Many indigenous species can adhere to intestinal cells, which helps maintain them in their specific niche. Whereas the indigenous types are permanent inhabitants, the transient types are either passing through or temporarily colonizing a site from where the specific indigenous type has been removed because of some inherent or environmental factors (such as antibiotic intake).

Among the indigenous microbial flora, several species of *Lactobacillus* in the jejunum and ilium, and *Bifidobacterium* in the large intestine, are thought to have beneficial effects on the health of the GI tracts of the hosts. From the intestines and intestinal content of humans, *Lactobacillus acidophilus, Lab. fermentum, Lab. rhamonosus, Lab. reuteri, Lab. casei, Lab. lactis, Lab leichmannii, Lab. plantarum, Bifidobacterium bifidus, Bif. longum, Bif. adolescentis, Bif. infantis*, and others have been

isolated. However, age, food habits, and health conditions greatly influence the species and their levels. There is some belief that a portion of the intestinal *Lactobacillus* species is transient. Initially, it was considered that *Lab. acidophilus, Lab. reuteri*, and some *Bifidobacterium* are the main indigenous species. At present, several other *Lactobacillus* species, such as *Lab. casei* and *Lab. rhamnosus,* have been tested to be beneficial. The presence of high numbers of the indigenous *Lactobacillus* species in the feces (and content of the large intestine) probably results from their constant removal from the small intestine.

III. IMPORTANT CHARACTERISTICS OF BENEFICIAL BACTERIA

Some relevant characteristics of *Lab. acidophilus, Lab. reuteri*, and *Bifidobacterium* species are briefly discussed here.[4–6] All three are found in the GI tract of humans as well as in animals and birds. They are Gram-positive rods and grow under anaerobic conditions. *Lab. acidophilus* is an obligatory homolactic fermentator; *Lab. reuteri* is a heterolactic fermentator and produces lactic acid, ethanol, and CO_2; and *Bifidobacterium* species produce lactic and acetic acids (in 2:3 ratio). They are less sensitive to stomach acid than many other bacteria under a given condition, and highly resistant to bile, lysozyme, and pancreatic enzymes present in the GI tract (small intestine). The two *Lactobacillus* species are present in low numbers in the jejunum, but in relatively high numbers in the ilium, especially toward the distal part, whereas *Bifidobacterium* species are present in the proximal part of the colon (near the ilium). All three are able to colonize in their respective niches, but studies with *Lab. acidophilus* reveal that all strains do not adhere to the GI mucosa of the host. Other studies have shown that the ability to adhere to the intestinal epithelial cells by *Lab. acidophilus* strains can be species specific, i.e., a specific strain can adhere to a particular species only and the trait may be lost during prolonged culturing under laboratory conditions.

Under normal conditions, these three species are thought to help in maintaining the ecological balance of GI tract microflora by controlling growth rate of undesirable microflora. This effect is produced through their ability to metabolize relatively large amounts of lactic and acetic acids. In addition, they can produce specific inhibitory substances, several of which are recognized. Many strains of *Lab. acidophilus* are known to produce bacteriocins, although they are most effective against closely related Gram-positive bacteria. Also, because of the sensitivity of bacteriocins to proteolytic enzymes of the GI tract, their actual role in controlling undesirable Gram-positive bacteria in the GI tract is disputable. Some strains also produce compounds that are not well characterized, but have been reported to have an antibacterial effect on both Gram-positive and Gram-negative bacteria. Some *Lab. reuteri* strains while growing in glycerol produce reuterine, which is inhibitory to both Gram-positive and Gram-negative bacteria. Antibacterial metabolite production by a few *Bifidobacterium* species is also reported. Some strains of *Lab. acidophilus* are able to deconjugate bile acids to produce compounds that are more inhibitory than the normal bile acids. Some *Lactobacillus* strains can also produce H_2O_2, but probably not under anaerobic conditions in the GI tract. Identification of specific antibacterial compounds other than acids

(such as reuterine) in these beneficial bacteria will help in understanding their role in maintaining intestinal health.

Several studies have indicated that beneficial effects of these bacteria are produced when they are present in relatively high numbers in the intestinal tract ($\geq 10^{6-7}$/g intestinal content). Diets rich in foods from plant sources, as opposed to those rich in foods from animal sources, seem to favor their presence in higher numbers. Many other conditions in a host also can reduce bacterial numbers in the GI tract, such as antibiotic intake, mental stress, starvation, improper dietary habits, alcohol abuse, and sickness and surgery of the GI tract. This, in turn, can allow the undesirable indigenous or transient bacteria to grow to high levels and produce enteric disturbances, including diarrhea, flatulence, and infection by enteric pathogens.

IV. BENEFICIAL EFFECTS OF PROBIOTICS

In the last 40 years, studies have been conducted to determine specific health benefits from the consumption of live cells of beneficial bacteria.[1,4–7] Live cells have been consumed from three principal sources: (1) as fermented milk products, such as yogurt, which contains live cells of *Lab. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* and is supplemented with *Lab. acidophilus* and others, and pasteurized milk, which contains *Lab. acidophilus*; (2) as supplementation of foods and drinks with live cells of one, two, or more types of beneficial intestinal bacteria, such as *Lab. acidophilus, Lab. reuteri, Lab. casei*, and *Bifidobacterium* species; and (3) as pharmaceutical products of live cells in the form of tablets, capsules, and granules. The beneficial effects from consuming these live cells were attributed to their ability to provide protection against enteric pathogens, supply enzymes to help metabolize some food nutrients (such as lactase to hydrolyze lactose) and detoxify some harmful food components and metabolites in the intestine, stimulate intestinal immune systems, and improve intestinal peristaltic activity. Some of these are briefly discussed here. In addition, many other benefits have been claimed by entrepreneurs, without any scientific basis, and they are not discussed.

A. Lactose Hydrolysis[1,4–6]

Lactose-intolerant individuals, because of a genetic disorder, are unable to produce lactase (β-galactosidase) in the small intestine. When they consume milk, lactose molecules are not hydrolyzed in or absorbed from the small intestine but passed to the colon. They are then hydrolyzed in the colon by lactase of different bacteria to glucose and galactose and then further metabolized to produce acids and gas, resulting in fluid accumulation, diarrhea, and flatulence. Consumption of yogurt, acidophilus milk, live cells of *Lactobacillus*, especially *Lab. acidophilus* in fresh milk and pharmaceutical products, reduces the symptoms in lactose-intolerant individuals. This benefit is attributed to the ability of beneficial bacteria to supply the needed lactase in the small intestine. However, as *Lab. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* do not survive stomach acidity well and are not normal intestinal bacteria, the benefit of consuming normal yogurt is considered to be due to the

reduced amounts of lactose in yogurt, as compared to milk, and to the supply of lactase from the dead cells. In contrast, the intestinal bacteria, especially some *Lactobacillus* species, could, under proper conditions, colonize the small intestine and subsequently supply lactase. Different studies, however, did not unequivocally prove the desired benefits, probably because of differences in study methods, including the use of strains that lack β-galactosidase, use of strains that are not host specific, use of species that are not of the intestinal type or unable to adhere to the intestine, use of preparations with low viable cells, and lack of expertise in microbiology and gastroenterology research.

B. Reducing Serum Cholesterol Level[1,4–6]

Consumption of fermented dairy products (some containing unknown microorganisms) and high numbers of live cells of beneficial intestinal bacteria has been associated with low levels of serum cholesterol in humans. This is attributed to two possible factors. One is the ability of some intestinal lactobacilli to metabolize dietary cholesterol, thereby reducing amounts absorbed in blood. The other possibility is that some lactobacilli can deconjugate bile salts and prevent their reabsorption in the liver. The liver, in turn, uses more serum cholesterol to synthesize bile salts and indirectly helps reduce cholesterol level in serum. However, results of several studies by different researchers do not always favor this hypothesis. Again, this can be due to differences in experimental design, such as use of strains that do not metabolize cholesterol or deconjugate bile acid, and other reasons described before.

C. Reducing Colon Cancer[1,4–6]

Many of the undesirable bacteria in the colon have enzymes that can activate procarcinogens, either present in food or produced through metabolism of undesirable bacteria, to active carcinogens that, in turn, can cause colon cancer. Beneficial intestinal bacteria, both *Lactobacillus* and *Bifidobacterium* species, by their ability to control growth of undesirable bacteria in the colon, can reduce the production of these enzymes. Also, beneficial bacteria, by increasing intestinal peristaltic activity, aid in regular removal of fecal materials. This, in turn, lowers the concentrations of the enzymes and carcinogens in the colon and reduces the incidence of colon cancer. Several studies have shown that oral consumption of large numbers of live cells of the beneficial bacteria reduces fecal concentrations of enzymes such as β-glucuronidase, azoreductase, and nitroreductase of undesirable colon bacteria. However, the relationship between reduction of these enzymes and reduction in colon cancer from the consumption of beneficial intestinal bacteria has not been studied, and thus contributions of these bacteria in controlling colon cancer are not clearly known. Animal studies have shown that formation of aberrant crypts, considered to be putative precancerous lesions, is reduced by consuming live cells of beneficial GI tract bacteria, especially bifodobacteria. Again, the results are not consistent, which could be due to the factors listed before.

D. Reducing Intestinal Disorders[1,4–6]

Under certain conditions, indicated before, the intestinal population of beneficial bacteria can be reduced. The undesirable bacteria in the intestine and some transient pathogens (such as enteric pathogenic bacteria and Rotoviruses) from the environment can then cause enteric disorders, including infection. Infection results from the invasion and growth of enteric pathogens in the intestine. Ingestion of large numbers of live cells of beneficial intestinal bacteria over a period of time was reported to reduce these problems. Both infants and adults on oral antibiotic therapy can develop diarrhea because of a loss of desirable bacteria in the intestine and an increase in undesirable pathogenic bacteria and viruses. It was suggested that beneficial bacteria, when consumed in large numbers, establish in the intestine and produce antibacterial compounds (acids, bacteriocins, reuterine, and others unknown), which, in turn, control the pathogens. Deconjugation of biles by beneficial species also produces compounds that are more antibacterial than the bile salts; this has also been suggested as a mechanism to control the growth of undesirable enteric bacteria. Probiotic bacteria also increase the specific immunoglobulins, reduce intestinal permeability, and normalize intestinal microflora. In many studies, the results were not always positive. This, again, could be due to the variation in study methods, as described before, including the use of strains without the specific trait.

E. Modulating Immune Response[1,4–7]

Limited studies have shown that intestinal microorganisms act on intestinal defense barriers and help regulate systemic and local immune response. This is more effective at an early age, during the development of lymphoid tissues in the gut. Normal establishment of GI tract flora at an early age helps develop immunity to oral administration of antigens associated with inflammatory reaction in the gut. Oral administration of probiotic gut flora also helps overcome some immune response caused by the undesirable gut microflora. This beneficial effect is produced possibly by changing intestinal permeability, altering gut microbiology, improving intestinal immunological barrier functions, and alleviating intestinal inflammatory response. These beneficial effects have not been observed in many other studies. Differences in experimental design, experimental methods, and analysis of the data can be the reasons for the controversy.

F. Reducing Allergic Diseases[1,4–7]

Establishment of normal gut flora, which starts after birth and continues up to two years of age, may be important in the development in later life of counterregulatory ability against several specific immune responses. The normal flora of the GI tract enter the body through food, water, air, and other environmental sources. Raising infants in an oversanitary environment and feeding semisterile processed foods may interfere with the establishment of normal microflora in the GI tract. This may cause the immune system of infants to develop inflammatory response to many food

antigens. Probiotics containing beneficial gut bacteria can have a suppressive effect to such reaction by stimulating the production of antiinflammatory cytokines and reducing allergic reaction in sensitive individuals. As the results are not consistent, this at present is regarded as only a theory.

G. Miscellaneous Benefits

Many other health benefits of probiotics have been claimed, such as prophylaxis against urinogenital infection, increased calcium absorption from the intestine, stimulation of endocrine systems, growth promotion, and prolongation of youth and life. Many of these are merely claims and are yet to be proven by proper scientific research.

V. SOME ASPECTS TO CONSIDER

The health benefit theory of fermented foods and beneficial intestinal bacteria is controversial. Although an association effect, i.e., some benefits from their consumption, cannot be denied, many studies have not been able to prove the benefits without doubts. As suggested before, the differences in study methods could be one reason for the differences in results. In designing these studies, several aspects have to be recognized. There are definitely differences in human responses, but there are also differences in bacterial responses. In selecting bacterial strains, the following considerations are important.[8–11]

A. Strain Variation

Beneficial strains differ in adherence ability and specificity (Figure 15.1). An adherent strain should probably be favored over a nonadherent strain. Also, strains adherent to humans should be preferred over strains adherent to other species. The selected strains should have a strong adherence property. The adherent property can be lost during long maintenance under laboratory conditions. Many studies have been conducted without even knowing the source and identity of the strains. In selecting a strain for a study, the following factors need to be considered.

B. Sensitivity to Stomach Acids

Survivability of strains to low stomach pH varies greatly. This effect can be reduced by either reducing stomach acidity with food or by using strains that are proven resistant to acid environment.

C. Viability and Injury of Cells

Cells of beneficial bacteria when frozen, dried, exposed to low pH, high salts, and many chemicals can die. Among the survivors, many can be injured and killed by

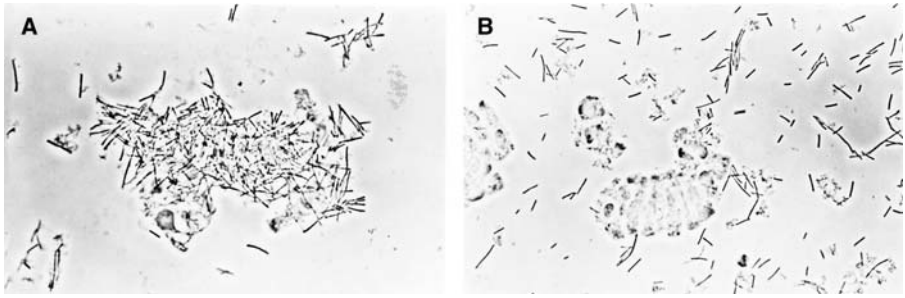


Figure 15.1 (A) Cells of an adherent strain of *Lab. acidophilus* are associated with the calf intestinal epithelial cells. (B) Cells of a nonadherent strain of *Lab. acidophilus* remain uniformly distributed and do not show adherence to epithelial cells from the same source.

stomach acid and bile salts and lysozyme in the intestine. In studies, it may be better to use cells grown for 16 to 18 h and maintained before feeding under conditions that retain their maximum viability.

D. Dose Level and Duration

Consumption of large numbers of live cells (10^9 per day), that are not stressed, over a period of time (ca. 14 d) is advocated to obtain benefit. Use of preparations that have low levels of viable cells, many of which could be stressed, cannot provide expected results. Many products that are currently marketed do not have the needed level of viable cells to produce beneficial effects (Table 15.1). Some products also have bacteria that are associated with improper sanitary practices.

E. Induced Lactase Trait

In *Lab. acidophilus*, lactase is an induced enzyme. To study the lactase effect, strains should be grown in lactose-containing media. In commercial preparations, a strain may be grown in glucose and thus not have lactase when consumed.

F. Antibacterial Substances

Many studies have reported that strains of beneficial intestinal bacteria produce metabolites that are active against many Gram-positive and Gram-negative bacteria. Some of these were identified, such as several bacteriocins, organic acid, and reuterine. Other substances need to be identified and examined in purified form for their antibacterial effectiveness.

G. True Species and Strains

Many species and strains used by many probiotic food producers do not have either proper identity or information of original sources. Also, many species that were previously regarded as *Lab. acidophilus* have been found to be different species,

Table 15.1 Viable Cell Counts (CFUs) of *Lab. acidophilus* in Commercial Probiotic Products

Product Type	CFUs/g[a]		Beneficial[b]
	Claimed	Actual	
Health Products (Dry)			
H1	Not specified	1.5×10^3 [c]	No
H2	1×10^8	1.6×10^3 [c]	No
H3	4×10^8	7.3×10^2 [c]	No
H4	Not specified	2.5×10^6 [c]	No
Pharmaceutical Products (Dry)			
P1	Not specified	4.8×10^6	Maybe
P2	Not specified	1.3×10^8	Yes
P3	5×10^9	3.2×10^9	Yes
P4	2×10^7	2.2×10^3	No

[a] Three samples from separate batches for each product type were enumerated for CFUs/ml in MRS-agar supplemented with 0.15% oxgall. Average results are presented. (From Marteau, P. and Rambaud, J.-E., *FEMS Microbiol. Rev.,* 12, 207, 1993.)

[b] A strain, with proven record of benefit, needs to be consumed at 10^{8-9} viable cells/day for the expected results. On that basis only, consumption of P2 and P3 (provided the strains are beneficial types) can be expected to produce benefit.

[c] The samples had (differed with products) coliform, lactose-negative Gram-negative rods, *Bacillus* spp., and cocci. This suggests unsanitary practices in the production of these products.

and many are not of intestinal origin. Before selecting a strain for a study, one needs to be sure, through testing by recommended methods, that the strain being used is what it is supposed to be.

H. Expertise in Research Areas

Lack of an understanding in research in the areas of microbiology, gastroenterology, immunology, oncology, and related fields may result in faulty experimental design and interpretation of data. In addition, differences in response by humans and animals in feeding trials can produce data of little value. Such studies are not expected to produce reproducible results.

Research conducted by considering these factors will help reduce bacterial variability. This, in turn, will help compare results of different studies and determine whether the health benefits of these bacteria are real or imaginary.

VI. CURRENT DEVELOPMENTS

Diverse types of probiotic products are now available in the market, and their consumption has gained popularity, especially in some European and South East Asian countries. Although a long list of health benefits are claimed by manufacturers, most have not been authenticated by well-designed scientific studies. In addition, as mentioned before, many products do not use species and strains that have been

studied for the possible beneficial effects and many products do not have high viable cell numbers required to produce benefit (Table 15.1). As a result, doubts about the beneficial effect of these products remain.

A. Standard of Identity[12]

Recently, there has been a concerted effort to develop a scientific basis for probiotic bacteria. Through a joint effort by scientists in this area, a standard of identity has been established for the beneficial probiotic bacteria, especially those from the genus *Lactobacillus*. This includes the ability to (1) adhere to GI epithelial cells; (2) interfere with adherence of enteric pathogens to GI epithelial cells; (3) persist and multiply in the GI tract without disturbing the normal microbial balance; (d) produce antibacterial metabolites; (e) coaggregate; (f) form a normal flora of the GI tract; and (g) be safe to the hosts. Following a critical evaluation of the data of scientific studies, five strains of lactobacilli are considered effective for use as probiotics: (1) *Lab. acidophilus* NCFM, (2) *Lab. casei* shirota, (3) *Lab. casei* CRL431, (4) *Lab. rhamonosus* GG, and (5) *Lab. reuteri* MM53. In the last few years, several other species/strains of *Lactobacillus* and *Bifidobacterium* have been included in the list.

B. Scientific Status Summary[13]

A scientific status summary has recently been published, listing the diverse species currently being used by probiotic product manufacturers and their claims for the bacterial species they are using. Some of the species include *Lab. acidophilus, Lab. bulgaricus, Lab. casei, Lab. fermentum, Lab. johnsonii, Lab. lactis, Lab. paracasei, Lab. plantarum, Lab. rhamonosus, Lab. reuteri, Bif. bifidus, Bif. longum, Bif. brevis, Streptococcus thermophilus,* and some yeasts. Their claims are not supported by valid scientific studies and have created disbelief among consumers. It was suggested in the report that, to overcome the current controversy, future research should be directed toward (1) identifying the phylogeny of a strain, (2) conducting health benefit studies with humans, (3) explaining the exact mechanisms by which a strain produces a benefit, (4) finding the dose level of viable cells and duration required for a benefit; (5) determining the ability of a strain to adhere to GI epithelial cells; (6) studying the influence of a probiotic strain on normal GI tract microflora, and (7) determining the proper and effective delivery systems of a probiotic strain.

There is no doubt that some bacterial species and strains do have beneficial influence on the health of the GI tract and probably the overall health of humans. But they have to be identified through valid scientific studies. The indiscriminate use, imaginative claims, and unscrupulous methods of a few have created the disbelief. A move for current collaborative scientific studies to define the standards of identity of probiotic strains, as well as the suggestions outlined in the status report regarding the research requirements before claiming a strain to have benefits, are the correct steps to remove the myths surrounding beneficial properties of probiotic bacteria.

C. Pathogenic Nature

In recent years, there have been a few reports on the isolation of lactic acid bacteria, such as *Pediococcus acidilactici* and *Lab. rhamonosus* strains, from infections in humans. Lactic acid bacteria, especially those used in food fermentation and as probiotics, are considered food grade and have been given the GRAS (generally regarded as safe) status internationally. In this regard, involvement of these bacteria in health hazards raised questions about their safety. The best answer to this doubt is an aphorism of the 16th-century German scientist Paracelsus: "All substances are poison — the right dose differentiates a poison and a remedy." From ancient civilization on, people have been consuming live cells of many of these bacteria through foods, without having major incidence of a health hazard, and they, without being eliminated, remained in our food chain. There are three possible reasons for the infections that result:

1. True identity of a strain is not known. Many strains currently used, especially as probiotics, have not been correctly identified to the species level (especially by genetic techniques), have not been tested for their beneficial properties to humans, or their sources have not been identified. The products may harbor pathogens because they have been produced under unsanitary conditions.
2. Being overly anxious for the benefit, an individual may consume a product in large volume. If the individual is immunocompromised, the large dose can cause a health hazard, not as a primary cause but maybe as a secondary cause.
3. Many isolates from infections have not been identified correctly to genus and species level by modern genetic techniques. By fermentation pattern only, it is difficult to identify the genus and species of an isolate in many situations.

The incidence of health hazard from beneficial bacteria, even with all the abuses, is very low. If we consider other beneficial things, such as use of antibiotics in the treatment of diseases, the incidence of health risk is much higher. Considering this, it is justifiable to assume that true food-grade lactic acid bacteria are safe.

D. Probiotics, Prebiotics, and Synbiotics[6,14]

1. Probiotics

The term *probiotics* was initially used with no specific definition. In 1989, Fuller[2] defined the term as products containing living microorganisms, which on ingestion (by humans, animals, and birds) in certain numbers exert health benefits beyond inherent general nutrition. In this statement, several requirements were included, such as the microbial cells should be alive and consumed in high numbers (usually 10^9 cells/d). But it did not include which microorganisms (only intestinal or others) should be used and consumed for how long (daily as a preventative or for 2 or more weeks as a curative). It indicates that the consumption should produce health benefit as judged by scientific clinical studies by reputed and independent research groups and results published in peer-reviewed journals (not just a claim by somebody). It also does not specify that the mechanisms by which the health benefits are produced

should be explained. In the status summary, as discussed before, these aspects have been specifically included.

In recent years, several studies have shown that some specific health benefits can be achieved by also consuming products containing dead cells or cell components of beneficial bacteria (e.g., immune modulation). It has to be decided in the future whether they also constitute probiotics.

2. Prebiotics

The beneficial effect of probiotic bacteria depends on their presence in high numbers in the GI tract. This can be achieved either by consuming a large number of viable cells of probiotic bacteria or by stimulating rapid growth of desirable gut bacteria by supplying appropriate nutrients. Among the beneficial gut bacteria, *Lactobacillus* species are predominant in the small intestine whereas *Bifidobacterium* species are predominant in the large intestine (colon). An approach has been taken to stimulate growth of *Bifidobacterium* in the colon by supplying one or more selective carbon and energy sources that are not metabolized by the bacteria in the small intestine as well as by many bacteria found in the colon. This gives *Bifidobacterium* a selective growth advantage and allows it to reach high numbers. These nutrients are termed *prebiotics* and defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity, or both, of one or a limited number of bacteria in the colon, that can improve the host's health. Some of the nutrients that have gained importance as prebiotics are lactulose, lactitol, fructooligosaccharides, galactooligosaccharides, lactosucrose, and α-inulin. Their actual effectiveness is currently being studied.

3. Synbiotics

The term *synbiotic* is coined to include both the terms *probiotics* and *prebiotics*. It is assumed that instead of using probiotics and prebiotics separately, a product containing both, i.e., the beneficial gut bacteria in high numbers as well as nutrient supplement for them will enable them to multiply rapidly in the gut and produce health benefit more effectively. This has to be studied to determine their actual effectiveness.

E. Biogenics[15]

The health benefit from the consumption of fermented dairy products is attributed to the lactic acid bacteria used in fermentation as well as the by-products of metabolism of milk nutrients by them. The latter aspect becomes important to explain health benefits attributed to fermented products in which nongut bacteria, such as *Lactococcus lactis, Str. thermophilus,* and *Lab. delbrueckii* ssp. *bulgaricus*, are used in fermentation. These products have recently been termed *biogenics* and include components in food that are derived through the microbial metabolic activity of food nutrients and have health benefits. One such group is the peptide produced by the exoproteinases of some lactic acid bacteria, such as *Lac. lactis* in buttermilk and

Lab. delbrueckii ssp. *bulgaricus* in yogurt. Some of the peptides thus produced and present in the fermented milk can reduce blood pressure in individuals with hypertension. This aspect is currently being studied.

F. Genome Sequence of Probiotic Bacteria[16]

The bacterial species and strains that are being studied for genome sequence include several *Lactobacillus* and *Bifidobacterium* species that are currently used as probiotic bacteria. These include *Lab. acidophilus, johnsonii, casei,* and *rhamnosus,* and *Bif. longum, breve* and *linens*. Once the complete genome sequences are obtained, they can be used to compare the genetic and functional diversities of the GI tract beneficial bacteria. This information can then be used in the future to better understand signaling processes among gut microbes, intestinal cells, food components, microbial metabolic products, and mechanisms of beneficial actions of probiotic bacteria. Finally, they will help design a better desirable gut bacteria or specific strains for specific beneficial effects.

VII. CONCLUSION

Although fermented foods and some lactic acid bacteria have been considered to help to normalize intestinal microbial ecosystems, there are doubts due to differences in results. These are due to improper uses of bacteria that have not been tested by well-designed studies by well-experienced research groups. Currently, standards of identity of beneficial intestinal bacteria are being advocated to change the myths to reality. In the future, genome sequence information will help better understand the interactions of food, intestinal systems, and microbial species and strains and use them effectively.

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QUESTIONS

1. Discuss the sequential establishment of GI flora in humans from birth to 2 years of age. How does the predominant flora differ between breast-fed and formula-fed babies?
2. Discuss the following terms, with examples: indigenous bacteria, transient bacteria, beneficial bacteria, and undesirable bacteria in the GI tract of humans.
3. List the important characteristics of beneficial bacteria present in the GI tract. Where are they present? What are some of the antibacterial substances they produce?
4. List the factors that could adversely affect the presence of beneficial bacteria in the human GI tract.
5. What is lactose intolerance? Explain the possible mechanisms by which consumption of some fermented dairy products or live cells of beneficial intestinal bacteria can help overcome this problem.
6. How can beneficial intestinal bacteria or some fermented foods possibly reduce serum cholesterol levels?
7. "Beneficial GI tract bacteria may be effective in reducing colon cancer and enteric diseases." What is the basis of this suggestion?
8. Experimental data do not always support the health benefits from the consumption of fermented foods and live cells of beneficial intestinal bacteria. This can be due to wide differences in experimental methods used. If you are planning to conduct such a study, what are some of the experimental factors you should consider?

CHAPTER 16

Food Biopreservatives of Microbial Origin

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I. INTRODUCTION

Even in the early days of food fermentation, our ancestors recognized that fermented foods not only have delicate and refreshing tastes, but also have a longer shelf life and reduce the chances of becoming sick from foodborne diseases. Fermentation helped them preserve some foods in fermented form longer than the raw materials,

which may be a major reason for food fermentation being so popular among early civilizations located in high-temperature zones. The knowledge of safety and stability of fermented foods has been transferred through the centuries and helped us understand their scientific basis.

It is now known that the food-grade bacteria associated with food fermentation can produce several types of metabolites that have antimicrobial properties, some of which are listed in Table 16.1.[1] There could be many more that are currently not identified. At present, there is an increased interest in the use of these antimicrobials in nonfermented foods to increase their stability and safety. Some of them, such as lactate and acetate (vinegar), have been used in many foods for a long time, whereas the others have generated much interest as potential food biopreservatives in place of some currently used nonfood preservatives such as nitrite, sulfite, parabens, diacetate, and ethylformate. Their effectiveness as food biopreservatives is discussed in this chapter. In addition, currently some yeasts have been found to inhibit the growth of molds in fruits and vegetables. This aspect is also briefly discussed.

II. VIABLE CELLS OF LACTIC ACID BACTERIA AS PRESERVATIVES

The process involves the addition of viable cells of mesophilic *Lactococcus lactis*, some *Lactobacillus* species, and *Pediococcus* species in high numbers to control spoilage and pathogenic bacteria during the refrigerated storage of a food at or below 5°C.[2] In the presence of mesophilic lactic acid bacteria, the growth of psychrotrophic spoilage and pathogenic bacteria is reported to be controlled. Growth of some of these spoilage and pathogenic bacteria at slightly higher temperatures (≅10 to 12°C) is also reduced. Studies were conducted by adding lactic acid bacteria to fresh meat, seafood, liquid egg, and some processed meat products, such as bacon, against *Clostridium botulinum, Salmonella* serovars, and *Staphylococcus aureus*. In refrigerated raw milk, meat, egg, and seafood, cells of *Lactobacillus, Lactococcus*, and *Leuconostoc* species were added to control the growth of psychrotrophic spoilage bacteria such as *Pseudomonas* spp. In some studies, growth of the psychrotrophs was inhibited by 90% or more during 4 to 10 d of refrigerated storage. Addition of cells of lactic acid bacteria in refrigerated raw milk also increased the yield of cheese and extended the shelf life of cottage cheese.

The inhibitory property can be because of the release of intracellular antimicrobial compounds, such as organic acids, bacteriocins, and hydrogen peroxide, from

Table 16.1 Antimicrobial Compounds of Food-Grade Bacteria

Metabolites	Effectiveness
Organic acids: lactic, acetic, propionic	Against bacteria and fungi
Aldehydes, ketones, and alcohols: acetaldehyde, diacetyl, ethanol	Against bacteria
Hydrogen peroxide	Against bacteria, fungi, and phages
Reuterine	Against bacteria and fungi
Bacteriocins	Against Gram-positive bacteria, normally

the cells by the nonmetabolizing lactic acid bacteria. The antibacterial role of hydrogen peroxide with the lactoperoxidase-thiocyanate system in raw milk is discussed later.

III. ORGANIC ACIDS, DIACETYL, HYDROGEN PEROXIDE, AND REUTERINE AS FOOD PRESERVATIVES

A. Organic Acids

In Chapter 11, the ability of starter-culture bacteria to produce lactic, acetic, and propionic acids was discussed. Commercially, lactic acid is produced by some *Lactobacillus* spp. capable of producing L(+)-lactate (or DL-lactate), acetic acid by *Acetobacter aceti*, and propionic acid by dairy *Propionibacterium* spp. They are in the generally regarded as safe (GRAS) list and are used in many foods as additives to enhance flavor and shelf life and as safety precautions against undesirable microorganisms. These acids and their salts are used in foods at ca. a 1 to 2% level[3,4] (also see Chapter 38).

Acetic acid, its salts, and vinegar (which contains 5 to 40% acetic acid and many other compounds that give it the characteristic aroma) are used in different foods for inhibiting growth and reducing the viability of Gram-positive and Gram-negative bacteria, yeasts, and molds. Acetic acid is generally bacteriostatic at 0.2% but bactericidal above 0.3%, and more effective against Gram-negative bacteria. However, this effect is pH dependent and the bactericidal effect is more pronounced at low pH (below pH 4.5). It is added to salad dressings and mayonnaise as an antimicrobial agent. It is permitted to be used as a carcass wash.

Propionic acid and its salts are used in food as a fungistatic agent, but they are also effective in controlling growth and reducing viability of both Gram-positive and Gram-negative bacteria. Gram-negative bacteria seem to be more sensitive at pH 5.0 and below, even at acid levels of 0.1 to 0.2%. Propionic acid is used to control molds in cheeses, butter, and bakery products and to prevent growth of bacteria and yeasts in syrup, applesauce, and some fresh fruits.

Lactic acid and its salts are used in food more for flavor enhancement than for their antibacterial effect, especially when used above pH 5.0. However, recent studies have shown that they have a definite antibacterial effect when used in foods at 1 to 2% levels, even at or above pH 5.0. Growth of both Gram-positive and Gram-negative bacteria is reduced, indicating increased bacteriostatic action. Below pH 5.0, lactic acid can have a bactericidal effect, especially against Gram-negative bacteria. It may not have any fungistatic effect in the food environment. It is used in many processed meat products and has also been recommended as a carcass wash.

The antimicrobial effect of these three acids is considered to be due to their undissociated molecules. The dissociation constants (pKa) are 4.8 for acetic, 4.9 for propionic, and 3.8 for lactic acid. Thus, at most food pH (5.0 and above), the undissociated fractions of the three acids can be quite low, the lowest being for lactic acid. The lower antimicrobial effectiveness of lactic acid is probably due to its low pKa. The antimicrobial action of the undissociated molecules is produced by

dissociation of the molecules in the cytoplasm following their entry through the membrane. H^+ released following dissociation initially reduces the transmembrane proton gradient and neutralizes the proton motive force, and then reduces the internal pH, causing denaturation of proteins and viability loss. However, other studies have suggested that these weak acids produce antimicrobial action through the combined effects of undissociated molecules and dissociated ions. The acids can also induce sublethal injury of the cells and increase their chance of viability loss. Undissociated molecules as well as dissociated ions can induce cellular injury.[3,4]

Several pathogenic bacterial strains, such as some strains of *Salmonella* Typhimurium, and *Escherichia coli* O157:H7, have been found to be relatively resistant to low pH due to their ability to overproduce some proteins induced by the acid environment. These proteins, also called stress proteins, enable the cells to withstand lower internal pH (see Chapter 9). The presence of such a strain in food, which is freshly acidified at a 1 to 2% level to control microorganisms, may pose a problem.

B. Diacetyl

Diacetyl is produced by several species of lactic acid bacteria in large amounts, particularly through the metabolism of citrate (see Chapter 11).[5] Several studies have shown that it is antibacterial against many Gram-positive and Gram-negative bacteria. Gram-negative bacteria are particularly sensitive at pH 5.0 or below. Diacetyl is effective at ca. 0.1 to 0.25%. Recent studies have shown that in combination with heat, diacetyl is more bactericidal than when used alone. Diacetyl has an intense aroma, and thus its use is probably limited to some dairy-based products in which its flavor is not unexpected. Also, it is quite volatile and thus may lose its effectiveness in foods that are expected to have a long storage life. Under reduced conditions, it is converted to acetoin, which may have reduced antibacterial effects. This will pose difficulties in its use in vacuum-packaged products. The antibacterial action is probably produced by deactivating some important enzymes. The dicarbonyl group (–CO–CO–) reacts with arginine in the enzymes and modifies their catalytic sites.[5]

C. Hydrogen Peroxide

Some lactic acid bacteria produce H_2O_2 under aerobic conditions of growth and, because of the lack of cellular catalase, pseudocatalase, or peroxidase, they release it into the environment to protect themselves from its antimicrobial action. Some strains can produce, under proper growth conditions, enough H_2O_2 to induce bacteriostatic (6 to 8 μg/ml) but rarely bactericidal action (30 to 40 μg/ml).[6] It is a strong oxidizing agent and can be antimicrobial against bacteria, fungi, and viruses (also bacteriophages). Under anaerobic conditions, very little H_2O_2 is expected to be produced by these strains. In refrigerated raw milk, the antibacterial action produced by adding nongrowing cells of mesophilic lactic acid bacteria is thought to be due to the ability of H_2O_2 of bacteria to activate the lactoperoxidase-thiocyanate system in raw milk.[7] Raw milk contains lactoperoxidase enzyme and

thiocyanate (SCN^-). In the presence of H_2O_2, lactoperoxidase generates a hypothiocyanate anion ($OSCN^-$), which at milk pH can be in equilibrium with hypothiocyanous acid (HOSCN). Both $OSCN^-$ and HOSCN are strong oxidizing agents and can oxidize the –SH group of proteins, such as membrane proteins of Gram-negative bacteria that are especially susceptible. This system is inactivated by pasteurization.

Hydrogen peroxide is permitted in refrigerated raw milk and raw liquid eggs (ca. 25 ppm) to control spoilage and pathogenic bacteria. Before pasteurization, catalase (0.1 to 0.5 g/1000 lb [455 Kg]) is added to remove the residual H_2O_2. Its antibacterial action is attributed to its strong oxidizing property and its ability to damage cellular components, especially the membrane. Because of its oxidizing property, it can produce undesirable effects in food quality, such as discoloration in processed meat, and thus has limited use in food preservation. However, its application in some food processing and equipment sanitation is being studied.

D. Reuterine

Some strains of *Lactobacillus reuteri*, found in the gastrointestinal tract of humans and animals, produce a small molecule, reuterine (β-hydroxypropionaldehyde; $CHO–CH_2–CH_2OH$), which is antimicrobial against Gram-positive and Gram-negative bacteria.[6] It produces an antibacterial action by inactivating some important enzymes, such as ribonucleotide reductase. However, reuterine is produced by the strains only when glycerol is supplied in the environment, which limits its use in food preservation. In limited studies, food supplemented with glycerol and inoculated with reuterine-producing *Lab. reuteri* effectively controlled growth of undesirable bacteria. Addition of reuterine to certain foods also effectively controlled growth of undesirable bacteria.[6]

IV. BACTERIOCINS OF LACTIC ACID BACTERIA AS FOOD PRESERVATIVES

The term *bacteriocin* is currently used to refer to a group of bioactive peptides produced by many bacterial strains from Gram-positive and Gram-negative groups. The bacteriocins produced by many strains of lactic acid bacteria and some propionic acid bacteria are of special interest in food microbiology because of their bactericidal effect normally to different Gram-positive spoilage and pathogenic bacteria and under stressed conditions to different Gram-negative bacteria important in food. Chemically, bacteriocin peptides are ribosomally synthesized, cationic, amphipathic, have α-helical or β-sheet structures, or both, and can have thioethers, disulfide bridges, or free thiol groups. The presence of an amphipathic α-helical structure with opposing polar and nonpolar faces along the long axis enables bacteriocins to interact with both the aqueous and lipid phases when bound to the surface of the membrane of a sensitive bacterial cell, leading to its functional destabilization and death of the cell. This aspect is discussed later.[8]

A. Bacteriocin-Producing Strains

Many bacteriocin-producing strains from the different genera and species of lactic acid bacteria have been isolated. Some of the species include *Lac. lactis, Streptococcus thermophilus, Lab. acidophilus, Lab. plantarum, Lab. sake, Lab. curvatus, Leuconostoc mesenteroides, Leu. carnosum, Leu. gelidium, Pediococcus acidilactici, Ped. pentosaceus, Ped. parvulus, Tetragenococcus halophilus, Carnobacterium piscicola, Enterococcus faecalis, Ent. faecium,* and *Bifidobacterium bifidum*. It appears now that bacteriocin-producing strains of lactic acid bacteria are quite common in the food environment. A proper procedure and experience in differentiating between a bacteriocin producer and a nonproducer (including one that produces other types of antibacterial compounds) are important to successfully isolate a bacteriocin-producing strain from the natural environment as well as identify a strain already present in culture collections. Some important factors to be considered in the isolation from the natural environment are boosting the number of producer cells over the associative microorganisms (such as incubating in low-pH broth), selecting sensitive strains of indicators (Gram-positive, preferably from the lactic acid bacterial group and more than one strain), using a known producer strain as a control, and recognizing the zone of growth inhibition around a colony on an agar-medium plate produced by a bacteriocin producer and a producer of other antibacterial compound (a circular clear zone for bacteriocins as opposed to a fuzzy zone for organic acids).[8]

Bacteriocins of lactic acid bacteria are bactericidal to sensitive cells, and death occurs very rapidly at a low concentration. Normally, Gram-positive bacterial strains are sensitive, the spectrum or range of which can vary greatly. Although Gram-negative bacterial cells are normally resistant to bacteriocins of lactic acid bacteria, they become sensitive following impairment of the cell surface lipopolysaccharide structure by physical and chemical stresses. Many bacteriocins are bactericidal against a few related species and strains, but several are effective against many strains from different species and genera. Some other general features of bacteriocins of lactic acid bacteria can be grouped as follows:[8–10]

- A producer strain is immune to its own bacteriocin but can be sensitive or resistant to other bacteriocins (e.g., pediocin AcH producing *Ped. acidilactici* is immune to pediocin AcH, sensitive to nisin A, and resistant to sakacin A).
- A strain can produce more than one bacteriocin (e.g., a *Lac. lactis* strain produces lactococcin A, B, and M); many strains of the same species can produce same or different bacteriocins (e.g., pediocin AcH is produced by many *Ped. acidilactici* strains, but sakacin A and sakacin P are produced by different strains of *Lab. sake*).
- Strains from different species and genera can produce the same bacteriocins (e.g., pediocin AcH is produced by strains of *Ped. acidilactici, Ped. pentosaceus, Ped. parvulus, Lab. plantarum, Bacillus coagulans*).
- Strains from different subspecies can produce different bacteriocins (e.g., different *Lac. lactis* ssp. *lactis* produce nisin A and lacticin 481).
- Different species from a genus can produce different bacteriocins (e.g., enterococcin EFS2 and enterocin 900 are produced by strains of *Ent. faecalis* and *Ent. faecium,* respectively).

- Natural variants of the same bacteriocin can be produced by different strains and species (e.g., nisin A and Z by *Lac. lactis* strains; pediocin AcH and coagulin by *Ped. acidilactici* and *Bac. Coagulans,* respectively).
- Many bacteriocins named differently before their amino acid sequences are determined can be the same (e.g., pediocin AcH and pediocin PA of *Ped. acidilactici* strains).[8,9]

B. Characteristics of Bacteriocins[9–14]

Although isolation of a large number of bacteriocins of lactic acid bacteria has been reported in the literature for most, the amino acids sequences have not been determined. Currently, amino acid sequences of 45 bacteriocins are known. These studies have shown that some of the bacteriocins that were initially given different names have the same amino acid sequences. Some examples are pediocin AcH and pediocin PA-1; curvacin A and sakacin A; and sakacin P and bavaricin A. In general, they contain less than 60 amino acids, but their bactericidal efficiency is not related to the number of amino acids in the molecule. They are cationic, and the net positive charge is higher at low pH. Because of their hydrophobic nature, the molecules have a tendency to aggregate, especially when stored in the liquid state and high concentrations. The bactericidal property is higher at lower pH, relatively stable at high temperature, and not affected by organic solvents. Anions in high concentrations can reduce bactericidal efficiency of some cationic bacteriocins by competitive exclusion. Different proteolytic enzymes can hydrolyze these peptides, leading to a loss of activity. They are fairly stable at frozen and refrigeration storage, but some with methionine can be oxidized to methionine sulfoxide, which reduces the potency (e.g., pediocin AcH). The monomers, especially with disulfide bonds, can form dimers and trimers by bond exchange, but retain the bactericidal efficiency.

Bacteriocins of lactic acid bacteria are characterized as ribosomally synthesized peptides as they undergo very little structural change following translation. In general, a molecule as translated is designated as prebacteriocin, which contains an N-terminus leader peptide and a C-terminus probacteriocin. The leader peptide is removed during transportation of the molecules from the cytoplasm side to outside through the membrane-bound ABC transporter; ABC transporter, acting as an endopeptidase, excises the leader peptide. Although, many bacteriocins have one peptide chain, some have two peptide chains. The function of the leader peptide is to direct the transport of a molecule through the ABC transporter. Once the bacteriocin molecules are released in the environment, depending on the pH, they either remain bound with the anionic molecules on the cell surface or are released in the environment. Recent studies have shown that some bacteriocins can be transported by the *sec*-dependent secretory system whereas a few do not have the leader sequence.

Bacteriocin molecules, based on the molecular structures, are subdivided into several groups. Broadly, they are grouped as Class I, which contains lanthionine rings, and Class II, which lacks lanthionine (Table 16.2). Lanthionine rings are formed after translation of prebacteriocin in the cytoplasm and before transportation through the ABC transporter by enzymatic dehydration of L-serine to dehydroalanine

Table 16.2 Classification of Bacteriocins of Lactic Acid Bacteria

Groups	Structural Basis	Bacteriocin Example	Producer Strain
Class I: (Lanthionine containing) Lantibiotics	5 Thioether rings	Nisin A	*Lac. lactis* ATCC11454
	3 Thioether rings	Lacticin 481	*Lac. lactis* CNRZ481
	2 Thioether rings	Lactocin S	*Lab. sake* L45
Class II: (no lanthionine)			
IIa: Cystibiotics (also pediocine-like)	4 Cysteines with 2 disulfide bonds	Pediocin AcH	*Ped. acidilactici* H
	2 Cysteines with 1 disulfide bond	Leucocin A	*Leu. gelidum* UAL 187
IIb: Two peptides[a]	2 Separate peptides	Plantaricin S	*Lab. plantarum* LCP 010
II: Nonsubgroup	Some contain thiol group (1 or more)	Carnobacteriocin A	*Car. piscicola* LV17A
II: No leader peptide[b]	Translated as probacteriocin	Enterocin L50	*Ent. faecium* L51
II: *sec*-Dependent[c]	Leader peptide recognizes *sec*-secretory system	Enterocin P31	*Ent. faecium* P13
Class III: Large molecules[d]	Heat labile	Helveticin J	*Lab. helveticus* J

[a] All other bacteriocins have one peptide.
[b] All other bacteriocins have leader peptides.
[c] All other bacteriocins are transported by specific ABC transporters.
[d] All other bacteriocins are heat stable.

and L-threonine to dehydrobutyrine and formation of thioether rings (Table 16.2 and Table 16.3). These bacteriocins, also designated as lantibiotics, may contain one or more thioether rings. In general, the bactericidal efficiency is higher in lantibiotics containing more thioether rings. Bacteriocins without lanthionine are grouped under Class II, currently containing five subgroups (Table 16.2). The subgroup cystibiotic has at present 14 bacteriocins and has two or four cysteines that form one or two disulfide rings in an oxidized environment (outside cytoplasm; Table 16.2). Cystibiotics with two disulfide rings have greater bactericidal effectiveness than those with one disulfide ring (Table 16.3). A bacteriocin with two peptides (that have some similarities in amino acid sequence with each other) needs both peptides for full bactericidal activity. However, each peptide has lower bactericidal effect. The bacteriocins under "nonsubgroup" in Class II do not have any common characteristics; however, a few can have a free thiol group (Table 16.3). A few bacteriocins, quite different from those described previously, are translated as probacteriocin (only the main part), without any leader peptide at the N-terminus region. Finally, several

Table 16.3 Thioether Rings, Disulfide Bonds, and Thiol Groups in Bacteriocins of Lactic Acid Bacteria

Bacteriocin	Structure
Nisin A:	I-dhB-Ala-I-dhA-L-Ala-Abu-P-G-Ala-K-Abu-G-A-L-M-G-Ala-N-M-K-Abu-A-Abu-Ala-H-Ala-S-I-H-V-dhA-K S: (3)–(7), (8)–(11), (13)–(19), (23)–(25), (28)–(34)
Pediocin AcH:	K-Y-Y-G-N-G-V-T-C-G-K-H-S-C-S-V-D-W-G-K-A-T-T-C-I-I-N-N-G-A-M-A-W-A-T-G-G-H-Q-G-N-H-K-C S: (9)–(14), (24)–(44)
Lactococcin B:	S-L-Q-Y-V-M-S-A-G-P-Y-T-W-Y-K-D-T-R-T-G-K-T-I-C-K-Q-T-I-D-T-A-S-Y-T-F-G-V-M-A-E-G-W-G-K-T-F-H SH: (24) (47)

bacteriocins of lactic acid bacteria are found to be secreted by the *sec*-dependent secretory mechanism, as opposed to ABC transporter systems of the other bacteriocins.

A comparison of the amino acid sequences of leader peptides revealed very little similarities among both Group I and Group II bacteriocins (Table 16.4). This is more evidenced with Group I bacteriocins. But some Group II bacteriocins have –G–G– at the C-terminus end of the leader peptide (at –1 and –2 positions). The double glycine is the recognition site for the endopeptidase activity of the ABC transporter to remove the leader peptide from the prebacteriocin molecule. Because a leader peptide propels the prebacteriocin molecule toward an ABC transporter in the membrane, it is possible that a specific amino acid sequence of a leader peptide recognizes a specific ABC transporter system for the most efficient transport of the probacteriocin (prebacteriocin without the leader peptide) in the environment; an interchange of leader peptides with other bacteriocins may not function very effectively. The amino acid sequences of probacteriocins (or bacteriocins) of lantibiotics have very little similarities (Table 16.5). In contrast, bacteriocins in the cystibiotic subgroup have several sequence homologies. The most important is the –YGNGV– sequence in the N-terminal halves of the molecules, which is known to have an important role in the bactericidal properties of the molecules. In addition, there are at least two cysteins usually at positions +9 and +14 that form a disulfide bond and are important for the bactericidal property. In several cystibiotics (e.g., pediocin AcH or PA-1, enterocin A) there are two more cysteine molecules in the C-terminal half of the

Table 16.4 Amino Acid Sequences of Leader Peptides of Bacteriocins of Lactic Acid Bacteria

Bacteriocin	Amino Acid Sequence
Nisin A (23)	MSTKDFNLDLVSVSKKDSGASPR
Lacticin 481 (24)	MKEQNSFLLQEVTESELDLILGA
Pediocin AcH (18)	MKKIEKLTEKEMANII**GG**
Leucocin A (24)	MMNMKPTESYEQLDNSALEQVV**GG**
Sakacin P (18)	MEKFIELSLKEVTAIT**GG**
Lactococcin A (21)	MKNQLNFNIVSDEELSEAN**GG**

Note: Numbers in parentheses are the number of amino acids. The last amino acid at the carboxyl end (right) is designated as –1. Class II bacteriocins have GG at positions –1 and –2.

Table 16.5 Amino Acid Sequences of Probacteriocins of Lactic Acid Bacteria

Probacteriocin	Amino Acid Sequence
Nisin A (34)	ITSISL**C**TPG**C**KTGALMG**C**NMKTAT**C**H**C**SIHVSK
Lacticin 481 (27)	KGGSGVIHTISHE**C**NMNSWQFVFT**CC**S
Pediocin AcH (44)	KY**YGNGV**T**C**GKHS**C**SVDWGKATT**C**IINNGAMAWATGGHQGNHK**C**
Leucocin A (37)	KY**YGNGV**H**C**TKSG**C**SVNWGEAFSAGVHRLANGGNGFW
Sakacin P (43)	KY**YGNGV**H**C**GKHS**C**TVDWGTAIGNIGNNAAANWATGGNAGWNK
Lactococcin A (55)	KLTFIQSTAAGDLYYNNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGLHH

Note: Numbers in parentheses are number of amino acids. The first N-terminal amino acid at the left is designated as +1. Y G N G V and cysteines in cystibiotics are indicated in bold letters.

molecule (at +24 and +44 positions in pediocin AcH or PA-1); the presence of an extra disulfide bond at the C-terminus makes the molecules more potent bactericidal agents than those with one disulfide bond at the N-terminus region (e.g., leucocin A). In general, the amino acid sequence of a bacteriocin determines the formation of α-helix and β-sheet domains in the molecule, which are important for its bactericidal efficiency.

C. Genetics and Gene Organization

The limited available information has revealed that the structural genes encoding prebacteriocin molecules of lactic acid bacteria can be encoded in a plasmid (e.g., pediocin AcH or PA-1), in the genome (e.g., sakacin P), or in a genome-integrated transposon (e.g., nisin A) of a producer strain. With plasmid-encoded bacteriocins, several variations are observed: a single plasmid can encode only one bacteriocin (e.g., pediocin AcH or PA-1) or more than one bacteriocin (e.g., lactococcin A, B, and M); the same bacteriocin can be encoded in different-size plasmids in the same species (e.g., lactococcin A in three separate strains of *Lac. lactis*) or different species (e.g., pediocin AcH in *Ped. acidilactici, Ped. pentosaceus, Ped. purvulus,* and *Lab. plantarum*); or a strain producing more than one bacteriocin that can be encoded in different plasmids (e.g., carnobacteriocin A and canobacteriocin B1 and B2 are encoded in two plasmids in a *Car. piscicola* strain).

In addition to the structural gene encoding for a bacteriocin, the DNA (plasmid or genome) also encodes several other genes related to its production (Figure 16.1). Some of them are quite simple (e.g., pediocin AcH) whereas others can be very complex (e.g., nisin A). There are many other differences in the organization of the genes associated with bacteriocin production in lactic acid bacteria. The genes could present in a single operon (e.g., in pediocin AcH and nisin A) or in more than one operon (sakacin P), and all genes are arranged in the same direction (e.g., sakacin P) or in opposite directions (e.g., leucocin A). In some, one or more genes can overlap (e.g., nisin A, leucocin A). The gene organization in the simplest form, as observed for pediocin AcH or PA-1 production, consists of an operon, with the promoter located upstream, and four genes, encoding for four proteins: *pap* or *ped A* for prepediocin, *pap* or *ped B* for immunity protein (Pap B or Ped B confer immunity against pediocin), *pap* or *ped C* for helper protein (Pap C or Ped C necessary for the efficient transport of pediocin), and *pap* or *ped D* (Pap D or Ped D for the ABC-transporter protein). Almost similar gene expressions, but with two promoters, are present in the production of leucocin A. In nisin A production, the genetic expression is quite different. Thirteen genes, *nis ABTCIPRKFEG*, are present in the operon, with the promoter located upstream and *nis A* encoding for prenisin (Figure 16.1). The functions of the other proteins from the other genes are as follows: Nis B and Nis C are involved in the dehydration of serine and threonine to dehydroalanine and dehydrobutyrine and thioether ring formation; Nis T is the ABC transporter involved in the transportation of nisin; Nis I is responsible for producer cell immunity to nisin; Nis P removes the leader peptide from prenisin following its transport by Nis T; Nis R and Nis K are regulatory

proteins and modulate transcription of *nis*-operon; Nis E, Nis F, and Nis G provide accessory protection to the producer cells against nisin to host. Similar genes for regulatory proteins are found in other bacteriocin operons such as sakacin P.

Biosynthesis of a bacteriocin involving transcription, translation, structural modification, and secretion occur in a concerted way. In pediocin AcH or PA-1 and similar bacteriocins, probably a single mRNA is produced for all four genes, which is then translated into four separate proteins (Figure 16.1). As soon as the 62-amino-acid prepedicin is translated in the cytoplasm, the leader peptide directs transport of the molecule outside the cell by the help of Pap or Ped D (ABC transporter) and C (helper). During transportation, the catalytic site of protein D recognizes –G–G– at the –1 and –2 positions of prepediocin and cleaves the leader peptide (18 amino acids); the transporter then secretes the 44-amino-acid pediocin in the environment. In an oxidized environment, the four cysteine molecules form two disulfide bonds, one at positions +9 and +14 and another at +24 and +44. The location and mechanism of the immunity protein Pap or Ped B in the producing cells are not known. In nisin A production, initially proteins Nis R and Nis K induce transcription of the cluster, probably to produce a single mRNA, which is then translated into 13 separate proteins. Nis B dehydrates serine and threonine to their respective dehydroamino acids, and Nis C with Nis B then enable dehydroamino acids to form thioether rings with cysteine residues in the molecule. The modified molecule is then translocated through the membrane by Nis T, and Nis P removes the 14-amino-acid leader peptide, releasing the 34-amino-acid nisin outside the cells. Nis E, Nis F, and Nis G provide extra protection to producer cells against nisin.

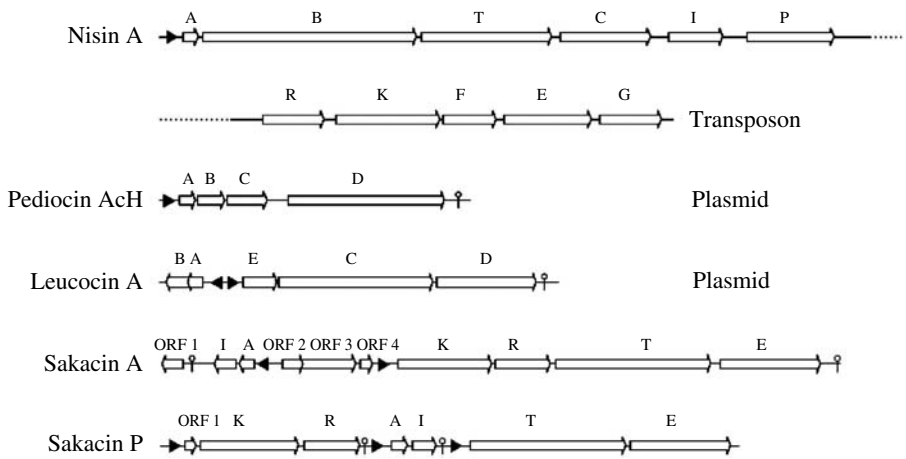


Figure 16.1 Organization of several well-characterized bacteriocin gene clusters. Structural genes are shown as open bars, promoters as black arrow heads, and rho-independent terminators as lollipop symbols. Genes of unknown functions are designated ORFs.

D. Mode of Action[9–14]

Bacteriocins of lactic acid bacteria kill sensitive bacterial cells very rapidly and the highly potent one at a very low concentration [minimum inhibitory concentration (MIC) ~ 0.01μg/ml for pediocin AcH to *Listeria monocytogenes*]. Their antibacterial actions can be summarized as follows:

1. They differ greatly in the spectra of antibacterial activity against sensitive Gram-positive bacteria (e.g., nisin and pediocin have wider spectra than leucocin or sakacin).
2. Their relative potency (MIC) against a sensitive strain differs greatly (e.g., pediocin is more potent than leucocin against *Lis. monocytogenes*).
3. Bactericidal efficiency of a bacteriocin increases at acidic pH, higher temperature, in the presence of a detergent, and against exponentially growing cells.
4. Even in the population of a most sensitive strain, there are variant cells that are resistant to a bacteriocin, but this property is not stable, because in the absence of the bacteriocin they become sensitive again.
5. A sensitive strain resistant to one bacteriocin can be sensitive to a second bacteriocin.
6. Gram-negative and resistant Gram-positive bacteria injured by a physical or chemical stress become sensitive to a bacteriocin.
7. Bacterial spores of a sensitive bacterium are resistant to a bacteriocin, but become sensitive following germination and outgrowth.

The bactericidal effect of a bacteriocin toward a sensitive bacterial cell is produced primarily by destabilization of the function of the cytoplasmic membrane. It is now accepted that, in general, the bacteriocin molecules are initially adsorbed on the membrane surface and form transient pores, leading to loss of protein motive force as well as the pH gradient across the membrane. This alters the permeability of the membrane, causing leakage of small nutrient molecules, as well as affecting the transport of nutrients and synthesis of ATP. These changes finally cause the cell to lose viability. In addition, some bacteriocins can cause lysis of sensitive cells. The exact mechanisms by which these changes are brought about differ with bacteriocins and are explained here by using nisin A and pediocin AcH or PA-1 as two examples. In the case of nisin, several molecules initially bind (dock) to the lipid II of the cell wall. This subsequently helps the molecules to come in contact with the membrane, leading to pore formation. Pore formation by nisin requires a voltage difference between the inside and outside of the membrane. Nisin is thus more potent against growing cells as opposed to resting cells of a target population. In contrast, the action of pediocin is not dependent on voltage difference of the membrane and is thus effective against both growing and resting cells. Pediocin also forms pores on the cytoplasmic membrane of target cells. As the molecules come in contact with the membrane, their random conformation changes to a defined structure. Several molecules then assemble in a cluster, leading to formation of a pore in the membrane.

E. Production and Purification

Bacteriocin production in lactic acid bacteria is directly related to the cell mass. Generally, the parameters that help generate more cell mass produce more bacteriocin molecules. The parameters include nutritional composition, initial and terminal pH and O–R potential of a broth, and incubation temperature and time. The species and the strains of a species growing under similar conditions differ greatly in the amount of bacteriocin production. In general, production of nisin A or pediocin AcH is much higher than leucocin or sakacin A. A nutritionally rich medium is always better, and, for many bacteriocins, growing the strains in a fermentor under a controlled terminal pH produces more bacteriocin. For example, nisin A production is much higher at pH 6.0, but pediocin AcH production is much higher at a lower terminal pH of 3.6. It is important to determine the optimum parameters for growth and bacteriocin production for an unknown strain to obtain high yield of bacteriocin.

Bacteriocin molecules following secretion by a producer strain remain either adsorbed on the cell surface or free in the medium, depending on the pH of the environment. More molecules remain free in the environment at pH 1.5 to 2.0, whereas at pH 6.0 to 7.0 they remain bound to the cell surface. Based on this pH-dependent adsorbtion–desorbtion bacteriocin on the cell surface of a producer strain, an effective and easy method has been developed to purify bacteriocin molecules in large amounts. A second method of purification that involves precipitation of the molecules by ammonium sulfate precipitation followed by stepwise gel filtration has also been used. This gives a highly purified preparation, but in small amounts.

F. Applications[8–10]

Bacteriocins of food-grade lactic acid bacteria are considered safe food biopreservatives and have the potential to use them to kill sensitive Gram-positive food spoilage and foodborne pathogenic bacteria. In foods that can contain injured Gram-negative bacteria, bacteriocins can also be effectively used to kill them. They are more effective when used in minimally heat-processed foods in suitable combinations of two or more (e.g., nisin and pediocin together). Table 16.6 presents the effectiveness of a preparation containing nisin and pediocin against spoilage pathogenic and spoilage bacteria in processed meat products during refrigerated storage. The results show that the bacteriocin preparation effectively reduced the Gram-positive spoilage (*Leu. mesenteroides*) and pathogenic (*Lis. monocytogenes*) bacteria as well as Gram-negative pathogens (*Salmonella* and *Esc. coli* O15:H7) during 6 weeks of storage at 4°C.

There are many other food products in which bacteriocins of lactic acid bacteria can be used effectively as preservatives (Table 16.7). Bacteriocins can also have a topical therapeutic use. However, before they can be used, research showing their effectiveness and safety has to be conducted. Finally, approval by regulatory agencies will be necessary before they can be used as food biopreservatives and topical therapeutic agents.

Table 16.6 Reduction in Viability and Growth of Food Spoilage and Foodborne Pathogenic Bacteria in Refrigerated Vacuum-Packaged Process Meats

			Log_{10} CFU/g Following Storage at 4°C		
Products[a]	**Test Bacteria**	**Treatment**	**1 d**	**2 to 4 wks**	**6 wk**
Hot dogs	*Leuconosoc mesenteroides*	Control	3.5	4.8	6.6
		Treated	1.0	<1.0[b]	<1.0
	Lactobacillus viridescens	Control	2.7	6.8	7.8
		Treated	<1.0	<1.0	<1.0
	Listeria monocytogenes	Control	3.4	NT	5.4
		Treated	<1.0	<1.0	<1.0
Roast beef	*Listeria monocytogenes*	Control	3.5	5.4	6.9
		Treated	<1.0	<1.0	<1.0
	Salmonella Typhimurium	Control	2.7	2.5	1.7
		Treated	2.3	1.8	<1.0
	Escherichia coli O157:H7	Control	3.2	3.0	2.5
		Treated	1.4	<1.0	<1.0
Turkey roll	*Leuconostoc mesenteroides*	Control	3.8	4.1	4.3
		Treated	3.4	1.8	1.5
Ham	*Listeria monocytogenes*	Control	2.9	3.2	5.4
		Treated	<1.0	<1.0	1.8

[a]Processed products were vacuum packaged and inoculated with cells of test strains with and without the bacteriocin mixture (pediocin + nisin in a 1:1 ratio to 5000 activity units/g and 1% lactate). The products were stored at 4°C and enumerated for survivors in suitable agar media.

[b]Two samples were used for each test and average results are presented. <1.0 means no CFU was obtained from 0.4 g equivalent of a product.

V. YEAST METABOLITES AS PRESERVATIVES

Certain yeasts, including strains of *Saccharomyces cerevisiae*, produce several proteins that have limited antimicrobial properties.[15] These proteins (designated as killer toxins or zymocins) can, through genetic manipulation, be altered to have wider antimicrobial spectrum, especially against fungi. However, very few studies are currently being conducted in this area.

Several yeast isolates normally present on the surface of fruits and vegetables are reported to prevent spoilage of the produce by molds. Some of the inhibitory compounds are small proteins, whereas others are enzymes. Cells of one such yeast isolate was found to adhere tightly with the mold mycelia and produce β-gluconase, which degrades the cell wall of the molds and kills them. Because many of these yeasts are normally present in fruits and vegetables that are eaten raw, they are not considered pathogenic, and thus can be used in place of fungicides to enhance the preservation of fruits and vegetables.[16]

VI. CONCLUSION

Food-grade bacteria and yeasts produce different antimicrobial compounds, such as organic acids, diacetyl, H_2O_2, reuterine, bacteriocins, and enzymes, that have bacteriostatic, bactericidal, fungistatic, and fungicidal action against microorganisms involved in food spoilage and foodborne diseases. They differ in the mode of antimicrobial action. Because they are from safe microorganisms, they can be used as food biopreservatives. Although some are currently used in a limited way, approval by regulatory agencies will be necessary for the use of others in food.

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QUESTIONS

1. List the antimicrobial compounds produced by different starter-culture bacteria.
2. Discuss the mode of antibacterial action of organic acids produced by starter-culture bacteria. At pH 6.0, why are propionic and acetic acids more antibacterial than lactic acid? List the advantages of using these acids in food preservation.
3. Describe the mode of antibacterial action of diacetyl and discuss why it has limited use as a food biopreservative.
4. Under what conditions can some lactic acid bacteria generate sufficient quantities of H_2O_2 to inhibit other microorganisms in a food system? What are some disadvantages of using H_2O_2 in food?
5. Discuss the advantages of using small amounts of H_2O_2 in raw milk to inhibit psychrotrophic bacteria such as *Pseudomonas* species. What needs to be done before pasteurizing this milk?
6. Define bacteriocins of lactic acid bacteria and list their four general characteristics.
7. How are bacteriocins grouped? Give one example of each group.
8. Define the terms prepeptide, propeptide, leader peptide, thioether rings, disulfide bonds, and thiol group in relation to the structure of bacteriocins.
9. List two important similarities in the amino acid sequence of cystibiotics that are related to their bactericidal property.
10. List three features of plasmid-encoded bacteriocins of lactic acid bacteria.
11. Briefly list differences in gene organizations associated with bacteriocin production in lactic acid bacteria.
12. Using either pediocin AcH or nisin A as an example, briefly explain the function of genes in the production of active molecules.
13. Briefly discuss how bacteriocin production of a producer strain of lactic acid bacteria can be enhanced.

14. List five potential applications of bacteriocins of lactic acid bacteria as food preservatives.
15. Briefly explain (a) potential therapeutic uses of bacteriocins and (b) use of antimicrobial properties of yeasts to preserve foods.

CHAPTER 17

Food Ingredients and Enzymes of Microbial Origin

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I. INTRODUCTION

Many microbial metabolites can be used as food additives to improve nutritional value, flavor, color, and texture. Some of these include proteins, essential amino acids, vitamins, aroma compounds, flavor enhancers, salty peptides, peptide sweeteners, colors, stabilizers, and organic acids. Because they are used as ingredients, they need not come only from microorganisms used to produce fermented foods but can be produced by many other types of microorganisms (also algae) with regulatory approval for safety before use in foods. Many enzymes from bacteria, yeasts, molds, as well as from plant and mammalian sources, are currently used for the processing of foods and food ingredients. Some examples are production of high-fructose corn syrups, extraction of juice from fruits and vegetables, and enhancement of flavor in cheese.

Recombinant DNA technology (or biotechnology) has opened up the possibilities of identifying and isolating genes or synthesizing genes encoding a desirable trait from plant and animal sources, or from microorganisms that are difficult to grow normally, clone it in a suitable vector (DNA carrier), and incorporate the recombinant DNA in a suitable microbial host that will express the trait and produce the specific additive or enzyme economically. In addition, metabolic engineering, by which a desirable metabolite can be produced in large amounts by a bacterial strain, is being used to produce food additives from new sources. The metabolites can then be purified and used as food additives and in food processing, provided they are safe to use generally regarded as safe (GRAS)-listed compound. Some of these aspects involving genetic manipulations are discussed in Chapter 12. The microbiology of the production and uses of some additives and enzymes are discussed here.

II. MICROBIAL PROTEINS AND FOOD ADDITIVES

This section discusses the different types of food additives that are and can be produced through microorganisms. Genetic and metabolic engineering are currently being studied to modify microorganisms that can economically produce many of the food additives. In the future, uses of food additives from these sources will increase. However, before they can be used in food systems, they have to be approved by regulatory agencies.

A. Single-Cell Proteins (SCPs)

Molds, yeasts, bacteria, and algae are rich in proteins, and the digestibility of these proteins ranges from 65 to 96%.[1,2] Proteins from yeasts, in general, have high digest-

ibility as well as biological values. In commercial production, yeasts are preferred. Some of the species used are from genera *Candida, Saccharomyces,* and *Torulopsis*. Some bacterial species have been used, especially from genus *Methylophilus*.

The use of microbial proteins as food has several advantages over animal proteins. There may not be enough animal proteins to feed the growing human population in the future, especially in many developing countries. Also, microbial proteins can be produced under laboratory settings. Thus, land shortage and environmental calamities (such as drought or flood) can be overcome. They can be produced on many agricultural and industrial wastes. This will help alleviate waste disposal problems and also reduce the cost of production. Microbial proteins can be a good source of B-vitamins, carotene, and carbohydrates.

There are some disadvantages of using microbial proteins as human food. They are poor in some essential amino acids, such as methionine. However, this can be corrected by supplementing microbial proteins with the needed essential amino acids. The other problem is that proteins from microbial sources can have high nucleic acid content (RNA and DNA; 6 to 8%), which, in the human body, is metabolized to uric acid. A high serum acid level can lead to kidney stone formation and gout. However, through genetic manipulations, the nucleic acid content in microbial proteins has been reduced.

Even though, at present, the use of microbial proteins as a protein source in human food is limited, they are being used as a protein source in animal feed. An increase of microbial proteins will automatically reduce the use of grains (such as corn and wheat) as animal feed, which then can be used as human food.[1,2]

B. Amino Acids

Proteins of most cereal grains are deficient in one or more of the essential amino acids, particularly methionine, lysine, and tryptophan. To improve the biological values, cereals are supplemented with essential amino acids. Supplementing vegetable proteins with essential amino acids has been suggested to improve the protein quality for people who either do not consume animal proteins (people on vegetarian diets) or do not have enough animal proteins (such as in some developing countries, especially important for children). To meet this demand as well as for use as nutrient supplements, large amounts of several essential acids are being produced. At present, because of economic reasons, they are mostly produced from the hydrolysis of animal proteins followed by purification.

In recent years, bacterial strains have been isolated, some of which are lactic acid bacteria that produce and excrete large amounts of lysine in the environment. Isolating high-producing strains of other amino acids, and developing strains by genetic and metabolic engineering that will produce these amino acids in large amounts, can be important for economical production of essential amino acids.[3]

C. Nutraceuticals and Vitamins

Many vitamins are added to foods and also used regularly by many as supplements. Thus, there is a large market for vitamins, especially some B vitamins and vitamins

C, D, and E. Some of these are obtained from plant sources, several are synthesized, and a few are produced by microorganisms. Vitamin C is now produced by yeast by using cheese whey. Microorganisms have also been a source of vitamin D. Many are capable of producing B vitamins.

The possibility of using gene-cloning techniques to improve production of vitamins by microorganisms may not now be very practical or economical. Vitamins are produced through multienzyme systems, and it may not be possible to clone the necessary genes. In recent years, through metabolic engineering, strains of lactic acid bacteria have been developed that when used in dairy fermentation produce high amounts of folate and some cyanocobalamin (B12) in the fermented products, thereby increasing the nutritional value of the products. In addition, strains of lactic acid bacteria have been developed that produce low-calorie sweeteners such as mannitol, sorbitol, and tagatose.[3]

D. Flavor Compounds and Flavor Enhancers

Flavor compounds and enhancers include those that are associated directly with the desirable aroma and taste of foods and indirectly with the strengthening of some flavors.[2–4] Many microorganisms produce different types of flavor compounds, such as diacetyl (butter flavor by *Leuconostoc*), acetaldehyde (yogurt flavor by *Lactobacillus acidophilus*), some nitrogenous and sulfur-containing compounds (sharp cheese flavor by *Lactococcus lactis*), propionic acid (nutty flavor by dairy *Propionibacterium*), pyrazines (roasted nutty flavors by strains of *Bacillus subtilis* and *Lac. lactis*), and terpenes (fruity or flowery flavors by some yeasts and molds). Some natural flavors from plant sources are very costly, because only limited amounts are available and the extraction process is very elaborate. By biotechnology, they can be produced economically by suitable microorganisms. Natural vanilla flavor (now obtained from plants), if produced by microorganisms, may cut the cost to only one tenth or less. Natural fruit flavors are extracted from fruits. Not only is it costly, but also large amounts of fruits are wasted. The possible production of many of these flavors by microorganisms through recombinant DNA technology is being studied.

Several flavor enhancers are now used to strengthen the basic flavors of foods. Monosodium glutamate (MSG; enhances meat flavor) is produced by several bacterial species, such as *Corynebacterium glutamicum* and *Micrococcus glutamicus*. Also, 5′ nucleotides, such as inosine monophosphate and guanosine monophosphate, give an illusion of greater viscosity and mouth feel in foods such as soups. They can be produced from *Bac. subtilis*.

Several small peptides such as lysylglycine have strong salty tastes. They can be produced by recombinant DNA technology by microorganisms and used to replace NaCl. Sweet peptides, such as monellin and thaumatins from plant sources, can also be produced by microorganisms through gene cloning. At present, the dipeptide sweetener aspartame is produced synthetically, but a method to produce it by microorganisms has been developed. By metabolic engineering, strains of lactic acid bacteria have been developed that can produce large quantities of diacetyl (for aroma of butter), acetaldehyde (for aroma of yogurt), α-ketoglutarate (to produce cheese flavor), and other compounds.[3]

E. Colors

Many bacteria, yeasts, and molds produce different color pigments. The possibility of using some of them, especially from those that are currently consumed by humans, is being studied.[4] This includes the red color pigment astaxanthine of a yeast species (*Phaffia* sp.). This pigment gives the red color to salmon, trout, lobster, and crabs. Another red pigment, produced by yeast *Monascus* sp., has been used for long in the Orient to make red rice wine. Because pigment production may involve multistep reactions, recombinant DNA techniques to produce some fruit colors by microorganisms may not be economical. However, they can be produced by the plant cell culture technique.

F. Exopolysaccharides (EPS)

Different polysaccharides are used in food systems as stabilizers and texturizers.[5,6] Although many of them are of plant origin, some are obtained from microbial sources. Strains of many lactic acid bacteria, such as *Streptococcus thermophilus, Lab. rhamnosus, Lab. helveticus, Lab. casei,* and *Lac. lactis*, produce many different types of exopolysaccharides (EPS) that contain units of glucose, galactose, rhamnose, mannose, and other carbohydrates. Many of these strains are currently being used to produce fermented dairy products with better consistency and texture (in yogurt and buttermilk), to hold moisture in low fat-high moisture cheeses (in mozzarella cheese). Dextran, an EPS produced by *Leuconostoc mesenteroides* while growing in sucrose, is used as a stabilizer in ice cream and confectioneries. Xanthan gum, produced by *Xanthomonas campestris*, is also used as a stabilizer. Introducing lactose-hydrolyzing genes in *Xanthomonas* species can enable it to grow in whey to produce the stabilizer economically.

G. Organic Acids

Production of lactic (by lactic acid bacteria), propionic (by propionic acid bacteria), and acetic (by acetic acid bacteria) acids and their different uses in foods are discussed in Chapter 11, Chapter 16, and Chapter 40. Several other organic acids and their salts are used in foods to improve taste (flavor and texture) and retain quality. Production of ascorbic acid by yeasts and its use as a vitamin supplement have also been discussed. Ascorbic acid is also used in some foods as a reducing agent to maintain color (to prevent color loss by oxidation). It also has an antibacterial action. Citric acid is used in many foods to improve taste and texture (in beverages) and stabilize color (in fruits). It also has some antibacterial property. Citric acid is produced by the mold *Aspergillus niger.*

H. Preservatives

Bacterial cells of lactic acid bacteria, several organic acids produced by them, and their bacteriocins can be used to control spoilage and pathogenic bacteria in foods. This aspect is discussed in Chapter 16.

III. MICROBIAL ENZYMES IN FOOD PROCESSING

Many enzymes are used in the processing of food and food additives.[7,8] About 80% of the total enzymes produced, on a dollar basis, is used by the food industries. Use of specific enzymes instead of microorganisms has several advantages. A specific substrate can be converted into a specific product by an enzyme through a single-step reaction. Thus, production of different metabolites by live cells from the same substrate can be avoided. In addition, a reaction step can be controlled and enhanced more easily by using purified enzymes. Finally, by using recombinant DNA technology, the efficiency of enzymes can be improved and, by immobilizing, they can be recycled. The main disadvantage of using enzymes is that if a substrate is converted to a product through many steps (such as glucose to lactic acid), microbial cells must be used for their efficient and economical production.

A. Enzymes Used

Among the five classes of enzymes, three are predominantly used in food processing: hydrolases (hydrolyze C–C, C–O, C–N, etc., bonds), isomerases (isomerization and racemization), and oxidoreductases (oxygenation or hydrogenation). Some of these are listed in Table 17.1 and their uses are discussed here.[7]

1. α-Amylase, Glucoamylase, and Glucose Isomerase

Together, these three enzymes are used to produce high-fructose corn syrup from starch. α-Amylase hydrolyzes starch at α-1 position randomly and produces oligosaccharides (containing three hexose units or more, e.g., dextrins). Glucoamylase hydrolyzes dextrins to glucose units, which are then converted to fructose by glucose isomerase.

α-Amylase is also used in bread-making to slow down staling (starch crystallization due to loss of water). Partial hydrolysis of starch by α-amylase can help reduce the water loss and extend the shelf life of bread.

2. Catalase

Raw milk and liquid eggs can be preserved with H_2O_2 before pasteurization. However, the H_2O_2 needs to be hydrolyzed by adding catalase before heat processing of the products.

3. Cellulase, Hemicellulase, and Pectinase

Because of their ability to hydrolyze respective substrates, the use of these enzymes in citrus juice extraction has increased juice yield. Normally, these insoluble polysaccharides trap juice during pressing. Also, they get into the juice and increase viscosity, causing problems during juice concentration. They also cloud the juice. By using these hydrolyzing enzymes, such problems can be reduced.

Table 17.1 Some Microbial Enzymes Used in Food Processing

Enzyme	Class[a]	Source	Substrate	Function/Use
α-Amylase	H	Bacteria, molds	Starch	Production of dextrins; brewing and baking
Catalase	OR	Molds	H_2O_2	Removal of H_2O_2; milk, liquid eggs
Cellulase	H	Molds	Cellulose	Hydrolyze cellulose; ethanol production, juice extraction
Glucoamylase	H	Molds	Dextrins	Dextrins to glucose
D-Glucose isomerase	I	Bacteria	Glucose	Glucose to fructose; high-fructose corn syrup
D-Glucose oxidase	OR	Molds	D-Glucose, oxygen	Flavor and color of liquid egg, juice
Hemicellulase	H	Bacteria, molds	Hemicellulose	Juice clarification
Invertase	H	Yeasts	Sucrose	Production of invert sugar
Lactase	H	Molds, yeasts	Lactose	Glucose or galactose from whey; low-lactose milk
Lipases	H	Bacteria, molds	Lipids	Cheese ripening
Pectinases	H	Molds	Pectin	Clarification of wine, fruit juice, juice extraction
Proteinases	H	Bacteria, molds	Proteins	Meat tenderization, cheese making and ripening

[a] H, hydrolases; I, isomerases; OR, oxidoreductases.

4. Invertase

Invertase can be used to hydrolyze sucrose to invert sugars (mixture of glucose and fructose) and increase sweetness. It is used in chocolate processing.

5. Lactase

Whey contains high amounts of lactose. Lactose can be concentrated from whey and treated with lactase to produce glucose and galactose. It can then be used to produce alcohol.

6. Lipases

Lipases may be used to accelerate cheese flavor along with some proteases.

7. Proteases

Different proteases are used in the processing of many foods. They are used to tenderize meat, extract fish proteins, separate and hydrolyze casein in cheese-making (rennet), concentrate cheese flavor (ripening), and reduce bitter peptides in cheese (specific peptidases).

B. Enzyme Production by Recombinant DNA Technology

The enzymes that are currently used in food processing are obtained from bacteria, yeasts, molds, plants, and mammalian sources. They have been approved by regulatory agencies, and their sources have been included in the GRAS list. There are some disadvantages of obtaining enzymes from plant and animal sources. The supply of these enzymes can be limited and thus costly. Also, molds grow slower than bacteria or yeast, and some strains can produce mycotoxins. It would be more convenient and cost-effective if the enzymes now obtained from nonbacterial sources (including yeasts, as their genetic system is more complicated than bacteria) could be produced in bacteria. This can be hypothetically achieved through recombinant DNA technology.[8] However, in trying to do so, one has to recognize that the bacterial host strains need to be approved by regulatory agencies if they are not in the GRAS list. Also, regulatory approval will be necessary for the source if it is not currently in the GRAS list.

The technique is rapidly going through many improvements. In brief, it involves separating specific mRNA (while growing on a substrate) and using the mRNA to synthesize cDNA by employing the reverse transcriptase enzyme. The cDNA (double stranded) is cloned in a suitable plasmid vector, which is then introduced by transformation in the cells of a suitable bacterial strain (e.g., *Esc. coli*). The transformants are then examined to determine the expression and efficiency of production of the enzyme. This method has been successfully used to produce rennin (of calf) and cellulase (of molds) by bacteria. Rennin thus produced is used to make cheese.

C. Immobilized Enzymes

Enzymes are biocatalysts and can be recycled. An enzyme is used only once when added to a substrate in liquid or solid food. In contrast, if the molecules of an enzyme are attached to a solid surface (immobilized), the enzyme can be exposed repeatedly to a specific substrate.[9] The major advantage is the economical use of an enzyme, especially if the enzyme is very costly.

Enzymes can be immobilized by several physical, chemical, or mechanical means. The techniques can be divided into four major categories (Figure 17.1).

1. Adsorption on a Solid Support

This technique relies on the affinity of the support for enzyme molecules. The technique involves adding an enzyme solution to the support (such as ion-exchange

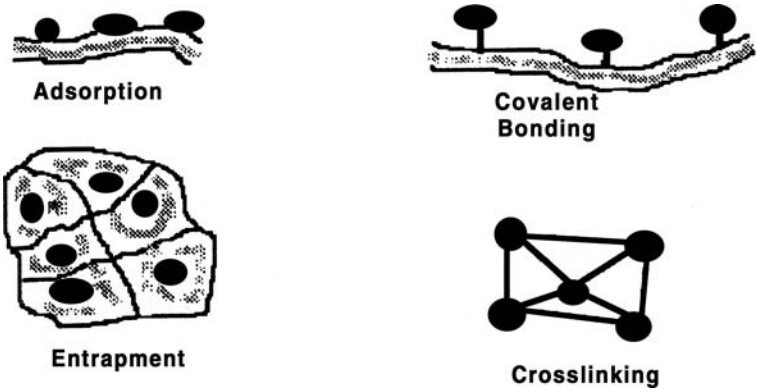


Figure 17.1 Schematic presentation of four methods of immobilization of enzymes.

resins) and washing away the unattached molecules. The association is very weak, and the molecules can be desorbed and removed.

2. Covalent Bonding

The enzyme molecules are covalently bound to a solid surface (such as porous ceramics) by a chemical agent. The enzyme molecules are accessible to the substrate molecules. The enzymes are more stable.

3. Entrapping

The enzyme molecules are enclosed in a polymeric gel (e.g., alginate) that has an opening for the substrate molecules to come in contact with the catalytic sites. The enzymes are added to the monomer before polymerization.

4. Crosslinking

Crosslinking is achieved by making chemical connections between the enzyme molecules to form large aggregates that are insoluble. This is a very stable system.

There are several disadvantages in enzyme immobilization. Immobilization can reduce the activity of an enzyme. Substrate molecules may not be freely accessible to the immobilized enzymes. The method may not be applicable if the substrate molecules are large. α-Amylase may not be a good candidate for immobilization because starch molecules, its substrate, are fairly large. However, glucose isomerase can be immobilized, as its substrate is small glucose molecules. The supporting materials can be contaminated with microorganisms that are difficult to remove and can be a source of contamination in food. The materials to be used as support should not be made of substances that are unsafe and should be approved by regulatory agencies. Some of the immobilized enzymes currently used are glucose isomerase, β-galactosidase, and aminoacylase.

Microbial cells can also be immobilized by the methods listed previously, and the techniques have been studied in the production of some food ingredients and beverages. Examples include *Asp. niger* (for citric acid and gluconic acid), *Saccharomyces cerevisiae* (for alcoholic beverages), and *Lactobacillus* species (for lactic acid).

D. Thermostable Enzymes

The term *thermostable enzymes* is generally used for those enzymes that can catalyze reactions above 60°C.[10] There are several advantages of using thermostable enzymes in a process. The rate of an enzyme reaction doubles for every 10°C increase in temperature; thus, production rate can be increased or the amount of enzyme used can be reduced. At high temperatures, when an enzyme is used for a long time (as in the case of immobilized enzymes), the problems of microbial growth and contamination can be reduced.

At high temperature, enzymes denature because of unfolding of their three-dimensional structures. The stability of the three-dimensional structure of an enzyme is influenced by the ionic charges, hydrogen bonding, and hydrophobic interaction among the amino acids. Thus, the linear sequences of amino acids in an enzyme greatly influence its three-dimensional structure and stability. Studies have revealed that increase in both ion pairing and hydrogen bonding on the surface of an enzyme (on three-dimensional structure) and increases in internal hydrophobicity increase the thermostability of an enzyme. For example, the enzyme tyrosinase from a thermolabile strain of *Neurospora* species denatures in 4 min at 60°C, but from a thermostable strain of the same species it denatures in 70 min at 60°C. An analysis of the amino acid sequences revealed that at position 96, tyrosinase has an aspargine (uncharged) in the thermolabile strain, but aspartic acid (charged) in the thermostable strain. Thus, an extra ionic charge (on the surface) increases the thermostability of this enzyme.

Several methods, such as chemical and recombinant DNA techniques, can be used to increase thermostability of an enzyme. Recombinant DNA technology can be used in two ways. If the enzyme is present in a thermostable form in a microorganism that is not in the GRAS list, the gene can be cloned in a suitable vector, which can then be introduced in a GRAS-listed microorganism and examined for expression and economical production. The other method is more complicated and involves determining the amino acid sequence of the enzyme and its three-dimensional structure (by computer modeling) to recognize the amino acids on the surface (or inside). The next step involves changing one or more amino acids on the surface to increase ionic or hydrogen bonding. This can be achieved by site-specific mutagenesis of base sequences of cDNA for the specific amino acid. The synthesized DNA can be incorporated in a vector and introduced in a desired microbial strain for expression of the enzyme and testing for its thermostability.

Several thermostable enzymes obtained from microorganisms on the GRAS list are currently being used. It is expected that in the future their production by different methods and use in food will increase.

E. Enzymes in Food Waste Treatment

Food industries generate large volumes of both solid and liquid wastes. Waste disposal methods have used different physical, chemical, and some biological methods.[11] Biological methods include anaerobic digestion and production of SCPs. Because of an increase in regulatory restrictions in waste disposal, effective and economical alternative methods are being researched. The possibility of using enzymes to reduce wastes and convert the wastes to value-added products is being developed. The availability of specific enzymes at low costs has been a major incentive in their use for waste disposal.

Some of the enzymes used in food waste treatments are polysaccharidases (cellulase, pectinase, hemicellulase, chitinase, and amylase), lactase, and proteinases. Treatment of fruits with cellulase and pectinase has increased juice yield and improved separation of solids from the juice. The solids can be used as animal feed. Chitinases are used to depolymerize the shells of shellfish, and the product used to produce SCPs. Amylases are used to treat starch-containing wastewater to produce glucose syrup for use in alcohol production by yeasts. Lactose in whey has been treated with lactase (β-galactosidase) to produce glucose and galactose, which are then used in alcohol production by yeast or to produce bakers' yeasts. Proteases are used to treat wastewater from fish and meat-processing operations. Some of these products are used as fish food.

In the future, development of better and low-cost enzymes through recombinant DNA technology will increase their uses in food waste treatment.

IV. CONCLUSION

The materials discussed in this chapter briefly summarize some of the cell components, metabolic end products, and enzymes produced by food-grade and regulatory-agency-approved microorganisms that are used in foods as additives to improve the nutritional and acceptance qualities of foods. Recent advances in genetic engineering and metabolic engineering of these bacteria have helped develop strains that can produce many unique products. As our knowledge on genome sequences and function of the genes of these strains increases, many new strains will be developed to produce other unique products. The future potential in this area is very high.

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QUESTIONS

1. What are the advantages and disadvantages of using microbial proteins as human food?
2. For each of the following, list one example from microbial origin that is used in food: amino acids, flavor compounds, flavor enhancers, color, polysaccharides (stabilizers), and nutraceuticals.
3. Briefly indicate how some lactic acid bacteria can be engineered to produce large quantities of diacetyl and acetaldehyde (see Chapter 12).
4. Name the microorganisms that are used to produce L(+)-lactic acid, acetic acid, and citric acid.
5. List the advantages and disadvantages of using microbial enzymes and microbial cells in food processing.
6. List five enzymes of microbial origin and discuss their specific uses in the food industry.
7. Discuss the advantages of using immobilized enzymes in the production of food ingredients. List three methods of immobilization.
8. What are the advantages of using a thermostable enzyme in the production of food ingredients (or a food)? How will recombinant DNA technology help in producing thermostable enzymes?
9. Discuss how microbial enzymes can be helpful in the treatment of wastes from food production.
10. "The potential of producing many types of food additives from food-grade microorganisms is very high." Briefly comment on the possibilities (you may use materials from other chapters in the section).

SECTION IV

Microbial Food Spoilage

A food is considered spoiled when it loses its acceptance qualities. The factors considered in judging the acceptance qualities of a food include color, texture, flavor (smell and taste), shape, and absence of abnormalities. Loss of one or more normal characteristics in a food is considered to be due to spoilage.

Food spoilage causes not only economic loss, but also loss of consumable foods. In the U.S. and some other countries where foods are produced and procured from many countries much more than the need, spoilage up to a certain level is not considered serious. However, in many countries where food production is not efficient, food spoilage can adversely affect the availability of food. With the increase in world population, serious consideration needs to be given on not only increasing food production but also reducing food spoilage, which for certain produce in some countries could reach 25% or more.

The acceptance qualities of a food can be lost because of infestation with insects and rodents, undesirable physical and chemical actions, and growth of microorganisms. An example of physical spoilage is dehydration of fresh vegetables (wilting). Chemical spoilage includes oxidation of fat, browning of fruits and vegetables, and autolytic degradation of some vegetables (by pectinases) and fishes (by proteinases). Microbial spoilage results either as a consequence of microbial growth in a food or because of the action of some microbial enzymes present in a food. In this section, food spoilage due to microbial growth and microbial enzymes is discussed under the following topics:

Chapter 18: Important Factors in Microbial Food Spoilage
Chapter 19: Spoilage of Specific Food Groups
Chapter 20: New Food Spoilage Bacteria in Refrigerated Foods
Chapter 21: Food Spoilage by Microbial Enzymes
Chapter 22: Indicators of Microbial Food Spoilage

CHAPTER 18

Important Factors in Microbial Food Spoilage

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I. INTRODUCTION

Microbial food spoilage occurs as a consequence of either microbial growth in a food or release of microbial extracellular and intracellular (following cell lysis) enzymes in the food environment. Some of the detectable parameters associated with spoilage of different types of foods are changes in color, odor, and texture; formation of slime; accumulation of gas (or foam); and accumulation of liquid (exudate, purge). Spoilage by microbial growth occurs much faster than spoilage by microbial extra- or intracellular enzymes in the absence of viable microbial cells. Between initial production (such as harvesting of plant foods and slaughter of food animals) and final consumption, different methods are used to preserve the acceptance qualities of foods, which include the reduction of microbial numbers and growth. Yet microorganisms grow and cause food spoilage, which for some foods could be relatively high. It is important to understand the factors associated with microbial food spoilage, both for recognizing the cause of an incidence and developing an effective means of control.

II. SEQUENCE OF EVENTS

Generally, for microbial food spoilage to occur, several events need to take place in sequence. Microorganisms have to get into the food from one or more sources; the food environment (pH, A_w, O–R potential, nutrients, inhibitory agents) should favor growth of one or more types of these contaminating microorganisms; the food must be stored (or abused) at a temperature that enables one or more types to multiply; and finally, the food must be stored under conditions of growth for sufficient length of time for the multiplying microbial types to attain the high numbers necessary to cause the detectable changes in a food. In a heat-treated food, the microorganisms associated with spoilage either survive the specific heat treatment (thermodurics) or get into the food following heating (as postheat contaminants). Spoilage of a heated food by microbial enzymes, in the absence of viable microbial cells, can result from some heat-stable enzymes produced by microorganisms in the foods before heat treatment. In addition, the foods need to be stored at a temperature for a sufficient length of time for the catalytic activities of the enzymes to occur to produce the detectable changes.

III. SIGNIFICANCE OF MICROORGANISMS

A. Microbial Types

Raw and most processed foods normally contain many types of molds, yeasts, and bacteria capable of multiplying and causing spoilage. (Viruses do not multiply in foods.) As multiplication is an important component in spoilage, bacteria (because of shorter generation time), followed by yeasts, are in favorable positions over molds to cause rapid spoilage of foods. However, in foods where bacteria or yeasts do not

grow favorably and the foods are stored for a relatively longer period of time, such as breads, hard cheeses, fermented dry sausages, and acidic fruits and vegetables, spoilage due to mold growth is more prevalent. Recent advances in anaerobic packaging of foods have also greatly reduced the spoilage of food by molds, and to some extent by yeasts, but not by anaerobic and facultative anaerobic bacteria. Thus, among the three microbial groups, the highest incidence of spoilage, especially rapid spoilage, of processed foods is caused by bacteria, followed by yeasts and molds.[1–4]

B. Microbial Numbers

To produce detectable changes in color, odor, and texture of a food accompanied with slime formation or gas and liquid accumulation, microorganisms (mainly bacteria and yeasts) must multiply and attain certain levels, often referred to as the "spoilage detection level." Although it varies with the type of foods and microorganisms, bacteria and yeasts need to grow and reach to ca. 10^7 cells/g, /ml, or /cm^2, of a food from the level present normally in a food. Depending on the specific nature of spoilage and microbial types, the spoilage detection level can range from $10^{6–8}$ cells/g, /ml, or /cm^2. Spoilage associated with H_2S, some amines, and H_2O_2 formation can be detected at a lower microbial load, whereas formation of lactic acid may be detected at a higher microbial load. Slime formation, associated with accumulation of microbial cells, is generally detected at $\geq 10^8$ cells/g, /ml, or /cm^2 of a food. It appears, then, that a food with relatively higher initial loads of spoilage bacteria (or yeasts) and a storage condition that favors rapid growth (shorter generation time) will spoil more rapidly than a food with a low initial load of microbes with longer generation time. In the hypothetical example (Figure 18.1), the population reached the spoilage detection level within 7 d with a high initial load (ca. 5×10^5/g) as opposed to 20 d with a low initial load (ca. 5×10^2/g) during storage at 12°C. However, when the product with a low initial load was stored at 4°C (to increase the generation time), it took ca. 55 d for the spoilage bacteria to reach the spoilage detection level. To reduce microbial spoilage of a food, one needs to aim at achieving both the low initial load and longer generation time of spoilage microorganisms during storage. It has to be recognized that the mere presence of 10^7 cells/g, /ml, or /cm^2 without growth (e.g., from a massive initial contamination) will not immediately cause a food to lose its acceptance quality; but such a food will spoil very rapidly following growth of the contaminants. Bioprocessed foods, in general, contain very high numbers of microorganisms ($10^{8–9}$ cells/g or /ml). However, under normal conditions, they are desirable types and the fermented foods are not considered spoiled. Spoilage of these foods can occur because of growth of undesirable bacteria, such as slime formation and off-flavor in cottage cheese by *Alcaligenes* and *Pseudomonas* spp. In such products, selective methods should be used to determine the populations of undesirable bacteria or yeasts.[2,3]

C. Predominant Microorganisms

The microbiological profile of a food is quite different from that of a pure culture growing in a laboratory medium. An unspoiled, nonsterile food generally contains

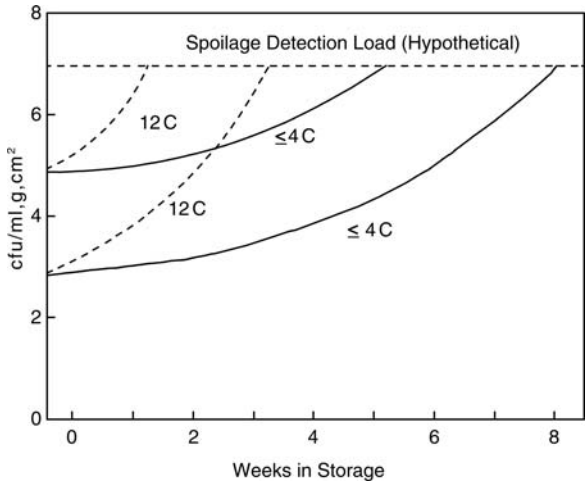


Figure 18.1 Graphical illustration showing the influence of initial bacterial levels and storage temperatures on the shelf life of a refrigerated product.

many types of microorganisms, such as bacteria, yeasts, and molds (also viruses) from different genera, maybe more than one species from the same genus, and even more than one strain from the same species. The population level of each type can vary greatly (Chapter 19). However, when the same food is spoiled, it is found to contain predominantly one or two types, and they may not even be present initially in the highest numbers in the unspoiled or fresh product. Among the different species initially present and capable of growing in a particular food, only those with the shortest generation time under the storage conditions attain the numbers rapidly and cause spoilage. In a study, a beef sample (pH 6.0) was found to initially contain ca. 10^3 bacterial cells/g, with relative levels of *Pseudomonas* spp. 1%, *Acinetobacter* and *Morexella* 11%, *Brochothrix thermosphacta* 13%, and others (*Micrococcus, Staphylococcus, Enterobacteriaceae*, lactic acid bacteria, etc.) 75%. Following aerobic storage at 2°C for 12 d, the population reached 6×10^7 cells/g, with the relative levels of *Pseudomonas* spp. 99% and all others 1%. Many of the bacterial species present initially could grow at the storage condition of the meat, but *Pseudomonas* spp. had the shortest generation time. As a result, initially even they constituted only 1% of the total population; after 12 d, they became predominant (99%). If the same meat sample were stored at 2°C anaerobically (such as in vacuum package) until the population had reached 10^7/g, the predominant bacteria would have been, with most probability, facultative anaerobic *Lactobacillus* or *Leuconostoc,* or both, because of their growth advantages.[1]

In this context, it is important to recognize that the generation time of a microbial species, even under optimum conditions of growth, is much longer in a food than in a microbiological broth. Also, under the same storage conditions, the growth behavior of a microbial mixed population can be quite different in a food as compared to a broth. Because of this, the predominant types obtained following growing an initial mixed microbial population from a food in a broth and in the same food under

identical conditions could be different. Although growing in a broth is convenient and, if properly designed, can provide valuable initial information, it is always better to have studies with specific foods.

IV. SOME IMPORTANT FOOD SPOILAGE BACTERIA

Theoretically, any microorganism (including microorganisms used in food fermentation and pathogens) that can multiply in a food to reach a high level (spoilage detection level) is capable of causing it to spoil. Yet, in reality, bacterial species from only several genera have been implicated more with spoilage of most foods. This is dictated by the bacterial characteristics, food characteristics, and the storage conditions (Chapter 2). The influence of some of these factors in determining predominant spoilage bacteria in foods is briefly discussed.[2,3] Pathogens are not included here.

A. Psychrotrophic Bacteria

As indicated before, psychrotrophic bacteria constitute the bacterial species capable of growing at 5°C and below, but multiply quite rapidly at 10 to 25°C and even at higher temperatures. Many foods are stored on ice (chilling) and in refrigerators, and some are expected to have a long shelf life (50 d or more). Between processing and consumption, they can be temperature abused to 10°C and higher. Psychrotrophic bacteria (also many yeasts and molds that are psychrotrophic) can cause spoilage in these foods. If the food is stored under aerobic conditions, psychrotrophic aerobes are the predominant spoilage bacteria. In foods stored under anaerobic conditions (also in the interior of a prepared food), anaerobic and facultative anaerobic bacteria predominate. If the food is given low-heat treatment and not exposed to postheat contamination during storage at low temperature, psychrotrophic thermoduric bacteria can cause it to spoil.

1. Some Important Psychrotrophic Aerobic Spoilage Bacteria

They include *Pseudomonas fluorescens, Pse. fragi*, other *Pseudomonas* species, *Acinetobacter, Moraxella,* and *Flavobacterium*. (Some molds and yeasts are included in this group.)

2. Some Important Psychrotrophic Facultative Anaerobic Spoilage Bacteria

They include *Bro. thermosphacta, Lactobacillus viridescens, Lab. sake, Lab. curvatus,* unidentified *Lactobacillus* spp.*, Leuconostoc carnosum, Leu. gelidum, Leu. mesenteroides,* unidentified *Leuconostoc* spp., some *Enterococcus* spp., *Alcaligenes* spp., *Enterobacter* spp., *Serratia liquifaciens*, some *Hafnia* and *Proteus* spp., and *Shewanella* (previously *Alteromonas*) *putrefaciens* (and some microaerophilic yeasts).

3. Some Important Thermoduric Psychrotrophs

They include facultative anaerobes, such as spores of *Bacillus coagulans* and *Bac. megaterium*, some strains of *Lab. viridescens*; and anaerobes, such as spores of *Clostridium laramie*, *Clo. estertheticum*, *Clo. algidicarnis*, *Clo. putrefaciens*, and unidentified *Clostridium* spp. The spores survive low-heat treatment. Following germination and outgrowth, the cells grow at low temperature.

When a food is temperature abused above 5°C (such as during transport or display in stores), some true mesophiles (growth temperature range 15 to 45°C, optimum 25 to 40°C) can also grow. However, at 10 to 15°C, psychrotrophs will generally grow much faster than these mesophiles.

B. Thermophilic Bacteria

By definition, the bacteria in this group grow between 40 and 90°C, with optimum growth at 55 to 65°C. Some high-heat-processed foods are kept warm between 50 and 60°C for a long period of time (at delis, fast-food establishments, and restaurants). Spores of some thermophilic *Bacillus* and *Clostridium* spp. can be present in these heat-treated foods, which at warm temperature germinate and multiply to cause spoilage. In addition, some thermoduric vegetative bacteria surviving low-heat processing (such as pasteurization) or thermophiles getting in food as postheat contamination can also multiply in these warm foods, especially if the temperature is close to 50°C. These include some lactic acid bacteria, such as *Pediococcus acidilactici* and *Streptococcus thermophilus*, as well as some *Bacillus* and *Clostridium* spp. They can also survive and cause spoilage of foods that are cooked at low heat (60 to 65°C as for some processed meats) or kept warm for a long time.

C. Aciduric Bacteria

Bacteria that can grow relatively rapidly in food at pH 4.6 or below are generally regarded as aciduric (or acidophilic). They are usually associated with spoilage of acidic food products such as fruit juices, pickles, salsa, salad dressings, mayonnaise, and fermented sausages. Heterofermentative lactic acid bacteria (such as *Lab. fructivorans, Lab. fermentum*, and *Leu. mesenteroides*) and homofermentative lactic acid bacteria (such as *Lab. plantarum* and *Ped. acidilactici*) have been associated with such spoilage. (Yeasts and molds are aciduric and thus are also associated with spoilage of such foods.)

V. SIGNIFICANCE OF FOODS

A. Food Types

Foods differ greatly in their susceptibility to spoilage by microorganisms. This is mainly because of their differences in intrinsic factors (A_w, pH, O–R potential, nutrient content, antimicrobial substances, and protective structures). A food with a

lower A_w (≅0.90) or a lower pH (≅5.3) is less susceptible to bacterial spoilage than one with A_w of ca. 0.98 or pH of ca. 6.4. However, molds and yeasts will probably grow equally well under both conditions. The influence of each of the intrinsic parameters on microbial growth has been described in Chapter 6. On the basis of susceptibility of spoilage, foods can be grouped as perishable (spoil quickly, in days), semiperishable (have a relatively long shelf life, few weeks or months), and nonperishable (have a very long shelf life, many months or years). In addition to intrinsic parameters, extrinsic parameters (storage conditions) play important roles in determining the ease of microbial spoilage of many foods.[3,4]

B. Food Nutrients

Microbial growth in a food is associated with the metabolism of some food carbohydrates, proteinaceous and nonprotein nitrogenous (NPN) compounds, and lipids. The influences of major types of carbohydrates (polysaccharides, trisaccharides, disaccharides, monosaccharides, and sugar alcohols), proteinaceous compounds (proteins, peptides), NPN compounds (amino acids, urea, creatine, trimethylamine oxide), and lipids (triglycerides, phospholipids, fatty acids, sterols) present in foods on microbial spoilage are briefly discussed here. The metabolic pathways of some of these compounds by microorganisms have also been discussed in Chapter 7 and Chapter 11. It is evident from previous discussions that microorganisms differ greatly in their abilities to metabolize different food nutrients (such as ability or inability to utilize cellulose and lactose as carbon sources, casein as nitrogen source, and oxidation of oleic acid). Similarly, the same nutrient (substrate) can be utilized by different microorganisms by different metabolic pathways to produce different end products (e.g., glucose metabolized by homolactic and heterolactic acid bacteria). The same nutrient (substrate) can be degraded to produce different end products under aerobic and anaerobic metabolism (respiration and fermentation, respectively). Thus, glucose is metabolized (catabolized) by *Micrococcus* spp. aerobically to produce CO_2 and H_2O, and by *Lab, acidophilus* anaerobically to produce mainly lactic acid. *Saccharomyces cerevisiae* metabolizes glucose aerobically to CO_2 and H_2O, but anaerobically to ethanol and CO_2. Under specific conditions, some microorganisms can also synthesize (anabolism) polymeric compounds as end products, such as dextran (polymer of glucose) production by *Leu. mesenteroides* while metabolizing sucrose. Some microorganisms can also secrete extracellular enzymes to break down large molecular nutrients (polymers) in a food (such as breakdown of starch by amylase produced by some molds). Finally, some microorganisms can synthesize pigments while growing in a food (such as *Micrococcus luteus* producing a yellow pigment).

Thus, metabolism of food nutrients during growth of microorganisms in a food can adversely change its acceptance quality in several ways. Some of the changes are odor (due to production of volatile end products), color (pigment production or oxidation of natural color compounds, such as oxidation of meat myoglobin), texture (breakdown of pectin by pectinases in vegetables, softening of the tissues in meat by proteinases, or thickening of milk by proteolytic enzymes), accumulation of gas (due to production of CO_2, H_2, or H_2S), formation of slime (due to production of

dextran and different types of exopolysaccharides or too many microbial cells resulting in confluent growth), and accumulation of liquid (purge accumulation; in fresh and processed meats due to breakdown of structures holding the water of hydration). Some of these changes also occur from the effect of microbial metabolites on food pH. Production of organic acids by microorganisms, causing lowering of food pH, can reduce the water-holding ability of food (such as growth of some lactic acid bacteria in low-fat, high-pH-processed meat products). Similarly, production of basic compounds by microorganisms in a food can shift its pH to the alkaline side and reduce its acceptance quality (such as decarboxylation of amino acids in some low-heat-processed meat products with the production of amines, shifting the pH to basic, and changing product color from light brown to pink in some processed meats; Chapter 20).

Table 18.1 lists some of the end products of microbial metabolism of food nutrients that are attributed to food spoilage. The end products vary with the nature of metabolism (e.g., aerobic respiration, anaerobic respiration, or fermentation; Chapter 7 and Chapter 11). It is evident that many of these metabolites are able to produce the changes associated with microbial food spoilage (change in odor, gas formation, or slime formation).[1–3]

C. Utilization of Food Nutrients

Almost all foods contain some amounts of carbohydrates, proteinaceous and NPN compounds, and lipids that are available for use by microorganisms during growth. However, the characteristics of food spoilage differ greatly because of differences in the nature and the amount of a specific nutrient present in a food, the type of microorganisms growing in the food, and the nature of metabolism (respiration or fermentation). In general, for energy production, microorganisms prefer to use metabolizable monosaccharides, disaccharides, and large carbohydrates first; followed by NPN, small peptides, and large proteinaceous compounds; and finally lipids. However, again metabolic characteristics depend on whether a particular species can use a specific carbohydrate (such as the ability or inability to utilize lactose) and the concentration of it present (limiting or high concentration). Also, with any nutrient, small molecules are used first before large molecules (polymers).

If a food has carbohydrates that can be fermented by the contaminating microorganisms, then it will usually be metabolized first. If the metabolizable carbohydrates are present in sufficient quantities, then the metabolic pathway remains

Table 18.1 Some End Products from Microbial Metabolism of Food Nutrients

Food Nutrient	End Products
Carbohydrates	CO_2, H_2, H_2O_2, lactate, acetate, formate, succinate, butyrate, isobutyrate, isovalerate, ethanol, propanol, butanol, isobutanol, diacetyl, acetoin, butanediol, dextran, levans
Proteinaceous and NPN compounds	CO_2, H_2, NH_3, H_2S, amines, keto-acids, mercaptans, organic disulfides, putrescine, cadaverine, skatole
Lipids	Fatty acids, glycerol, hydroperoxides, carbonyl compounds (aldehydes, ketones), nitrogenous bases

unchanged during rapid growth. However, if the carbohydrates are present in limited concentrations, then after the carbohydrates are used up the microorganisms usually start using NPN, small peptides, and other proteinaceous compounds. For example, yeasts growing in a fruit juice containing relatively high amounts of metabolizable carbohydrates (fructose, glucose, sucrose) will produce either CO_2 and H_2O (aerobically) or alcohol and CO_2 (anaerobically). However, *Pse. fluorescens* growing aerobically in fresh meat with limited amounts of glucose will first metabolize it and then start metabolizing free amino acids and other NPN compounds. If it is allowed to grow for a long time, it will produce extracellular proteinases to break down meat proteins to produce small peptides and amino acids for further metabolism. With time, it may even be able to produce lipases to break down meat lipids and use up some fatty acids. In a food (such as milk) containing large amounts of both carbohydrates (lactose) and proteins, a lactose-metabolizing microorganism will preferentially utilize the lactose and produce acid or acid and gas (*Lac. lactis* will produce lactic acid and *Leuconostoc* spp. will produce acid and gas), but a microorganism unable to utilize lactose will use the NPN and proteinaceous compounds for growth (*Pseudomonas* spp.). The spoilage patterns of these bacteria will be quite different.

In a mixed microbial population, as present normally in a food, availability and amount of metabolizable carbohydrates greatly affect the spoilage pattern. Fresh meats, because of a low level of glucose, are susceptible to spoilage through microbial degradation of NPN and proteinaceous compounds. However, if a metabolizable carbohydrate (such as glucose, sucrose, or lactose) is added to meat, metabolism of carbohydrates will predominate. If lactic acid bacteria are present as natural microflora and the growth environment is favorable, they will produce enough acids to arrest the growth of many normal microflora that preferentially metabolize NPN and proteinaceous compounds (e.g., Gram-negative pyschrotroph). This is commonly known as the protein-sparing effect (proteins are not metabolized). In the formulation of many processed meat products, metabolizable simple carbohydrates (usually glucose) are used to produce the protein-sparing effect.[1,2]

D. Microbial Growth in Succession

Intrinsic and extrinsic factors or environments of a food dictate which, among the mixed microbial species normally present, will multiply rapidly and become predominant to cause spoilage. However, as the predominant types grow, they produce metabolites and change the food environment. In the changed environment, some other species, initially present but previously unable to compete, may be in a favorable position to grow rapidly and again change the food environment further to enable a third type to grow rapidly. If sufficient time is given, the predominant microbial types and the nature of spoilage of a food can change. Sequential growth of *Lactococcus* spp., aciduric lactose-negative *Bacillus* sp., and Gram-negative rods (such as *Pseudomonas* spp.) in a milk sample can be used as a hypothetical example. Initially, rapid growth of *Lactococcus* spp. (able to metabolize lactose) under a favorable growth condition will reduce the pH from original 6.5 to 5.0 and reduce the growth rate of many other microbial species present. As the pH drops below 5.0,

the generation time of *Lactococcus* spp. becomes longer. However, *Bacillus* sp., because of its aciduric nature, can then start multiplication, metabolize proteins, and increase the pH (say 5.8). In the high pH, the *Pseudomonas* spp. present initially can then grow by metabolizing NPN and proteinaceous compounds and increase the pH further by producing basic metabolites (amines, NH_3). This way, the predominant spoilage microorganisms and the metabolites associated with spoilage (e.g., nature of spoilage) of a food can change if a food is stored for a fairly long time.[3]

VI. CONCLUSION

Growth of microorganisms in food to a high level causes detectable changes in the quality of food, which is generally termed as spoilage. Spoilage characteristics differ with the differences in microbial type and the food component being metabolized. Different aspects of food spoilage are discussed in this chapter. In Chapter 19, spoilage of specific food groups, the predominant microorganisms involved, and the nature of spoilage are discussed.

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QUESTIONS

1. Describe when a food is considered spoiled by microorganisms. You put a packet of lunchmeat in the refrigerator 2 to 3 weeks back and forgot about it. When you opened it, you thought it had a slightly sour odor. You asked your friends to smell it, but they said it smelled fine. What could be the reasons for this difference?
2. You opened a 2-l bottle of soft drink and kept it in the refrigerator and forgot about it. After 2 weeks, you found some molds growing on the surface of the liquid. Describe the sequence of events that resulted in this spoilage.
3. Discuss the significance of microbial types and numbers in food spoilage.
4. A food is usually spoiled by limited (usually one or two) types of microorganisms among the many types initially present. Describe how this predominance is established.
5. At present, psychrotrophic bacteria seem to be the most important spoilage bacteria. Suggest the possible reasons.

6. How can psychrotrophic bacteria be arbitrarily grouped? Give examples of two bacterial species from each group. Also, give two examples each of thermoduric, thermophilic, and aciduric spoilage bacteria.
7. Define aerobic respiration, anaerobic respiration, and fermentation. List four metabolites (for each) associated with (a) odor and (b) gas formation from food nutrients.
8. Discuss the importance of preferential nutrient metabolism by microorganisms and the nature of food spoilage from this, using meat and milk as examples.

CHAPTER 19

Spoilage of Specific Food Groups

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I. INTRODUCTION

The predominant microbial types normally expected to be present in different food groups have been listed in Chapter 2 and Chapter 4. Initially, a food produced under proper sanitary conditions generally contains microorganisms at a level (per g, ml, or cm^2) much lower than that at which spoilage is detected. Subsequently, growth of some of the microbial species among those initially present enables the microorganisms to reach the spoilage detection level. Many factors dictate which species will multiply relatively rapidly to become the predominant spoilage microorganisms. Along with microbial types, food types and food environments (both intrinsic and extrinsic factors) have important roles in determining the predominant spoilage microflora in a food. These aspects have been discussed in Chapter 4 and Chapter 6. In this chapter, predominant microorganisms associated with the spoilage of different food groups are described. As the foods in each group have similarities in nutrient content and the environment, similar microbial types are generally associated with spoilage. Pathogens are not included.

II. FRESH AND READY-TO-EAT MEAT PRODUCTS

A. Raw Meat

Fresh meats from food animals and birds contain a large group of potential spoilage bacteria that include species of *Pseudomonas, Acinetobacter, Moraxella, Shewanella, Alcaligenes, Aeromonas, Escherichia, Enterobacter, Serratia, Hafnia, Proteus, Brochothrix, Micrococcus, Enterococcus, Lactobacillus, Leuconostoc, Carnobacterium*, and *Clostridium*, as well as yeasts and molds.[1–6] The predominant spoilage flora in a meat is determined by nutrient availability, oxygen availability, storage temperature, pH, storage time of the product, and generation time of the

microorganisms present in a given environment. Postrigor meats are rich in nonprotein nitrogenous (NPN) compounds (ca. 13 mg/g; amino acids and creatine), peptides, and proteins, but contain low concentrations of carbohydrates (ca. 1.3 mg/g; glycogen, glucose, glucose-6-phosphate), with a pH ca. 5.5 and A_w more than 0.97. Dark, firm, dry (DFD) meats have almost no carbohydrates and a pH 6.0 or above.[1–5]

To delay microbial spoilage, fresh meats are stored at refrigerated temperature (≤5°C), unless the facilities are not available. Thus, normally psychrotrophic bacteria are the most predominant types in raw meat spoilage. Under aerobic storage at low temperature, growth of psychrotrophic aerobes and facultative anaerobes is favored. In retail-cut meats, because of a shorter generation time, *Pseudomonas* spp. grows rapidly, using glucose first and then amino acids; the metabolism of amino acids is accompanied by the production of malodorous methyl sulfides, esters, and acids. In meats with high pH or low glucose content, or both, *Acinetobacter* and *Morexella*, which preferentially metabolize amino acids instead of glucose, can grow rapidly and produce undesirable odors. Spoilage by these strict aerobes in the form of off-odor is detected at a population of ca. $10^{7–8}$ cells/cm^2 and slime at ca. $10^{8–9}$ cells/cm^2. The oxygenated red color of myoglobin undergoes oxidation to produce gray or brown metmyoglobin. DFD meats are spoiled more rapidly, inasmuch as the bacteria utilize amino acids immediately because of the absence of carbohydrates.

Refrigerated meat in a modified atmosphere, such as in a mixture of CO_2 and O_2, favors growth of facultative anaerobic *Brochothrix thermosphacta*, especially in meat with pH 6.0 or higher (DFD meat). It metabolizes glucose to acetic acid and acetoin, and leucine and valine to isovaleric and isobutyric acids, to produce off-odor (cheesy odor). Under anaerobic conditions, it metabolizes glucose to produce small amounts of lactic acid (which is not considered a cause of spoilage).

Psychrotrophic facultative anaerobes and anaerobes can grow in vacuum-packaged meat to produce different types of spoilage. *Lactobacillus curvatus* and *Lab. sake* metabolize glucose to produce lactic acid and the amino acids leucine and valine to isovaleric and isobutyric acids. These volatile fatty acids impart a cheesy odor in meat at a population level of more than $10^{7–8}$ cells/cm^2. This spoilage is not considered to be highly undesirable, because after opening the package, the odor disappears. However, when they metabolize cysteine and produce H_2S gas, the products have undesirable odor and color. Heterofermentative *Leuconostoc carnosum* and *Leu. gelidum* produce CO_2 and lactic acid, causing accumulation of gas and liquid in the package. *Shewanella putrefacience*, which can grow under both aerobic and anaerobic conditions, metabolizes amino acids (particularly cysteine) to produce methylsulfides and H_2S in large quantities. Along with offensive odors, they adversely affect the normal color of meats. H_2S oxidizes myoglobin to a form of metmyoglobin, causing a green discoloration. Facultative anaerobic *Enterobacter, Serratia, Proteus,* and *Hafnia* species metabolize amino acids while growing in meat to produce amines, ammonia, methylsulfides, and mercaptans, and cause putrefaction. Some strains also produce H_2S in small amounts to cause greening of the meat. Because amines and ammonia are produced, the pH of the meat usually changes to alkaline range, and meat can have a pinkish to red color. Psychrotrophic *Clostridium* spp., such as *Clostridium laramie*, have been found to cause spoilage associated with proteolysis and loss of texture of meat, accumulation of liquid in the bag, and

offensive odor, with an H_2S smell predominating. The color of the meat becomes unusually red initially and then changes to green (due to oxidation of myoglobin by H_2S). Some *Clostridium* spp., and probably *Enterococcus*, can cause spoilage of beef rounds and ham deep near the bone designated as bone sour or bone taint.

Comminuted meats spoil more rapidly than retail cuts because they have more surface area. Under aerobic storage, growth of aerobic bacteria (predominantly *Pseudomonas* spp.) causes changes in odor, texture, color, and sliminess. The inside is initially microaerophilic (due to dissolved oxygen from trapped air), which then changes to anaerobic, and growth of facultative bacteria predominates. In vacuum-packaged products, growth of lactic acid bacteria predominates in the initial stage. Heterofermentative lactic acid bacteria can cause accumulation of gas in the package. Some lactic acid bacteria also grow by utilizing amino acids. When the glucose is used up, Gram-negative facultative anaerobes grow and degrade amino acids to produce putrid odor.

To reduce spoilage of fresh meats, initial microbial level should be reduced. In addition, storage at low temperatures (close to 0 to –1°C), modified atmosphere packaging, and vacuum packaging should be done. Several other methods to reduce initial microbial load and slow growth rate of Gram-negative rods are being either used or tested. These include the addition of small amounts of organic acids to lower the pH of meat (slightly above pH 5.0), drying of meat surfaces (to reduce A_w), and a combination of the factors given previously, including lower storage temperature.

B. Ready-to-Eat Meat Products

This group includes high-heat-processed and low-heat-processed uncured and cured meat products. High-heat-processed cured and uncured meats are given heat treatment to make them commercially sterile. Thus, they may only have some thermophilic spores surviving, which will not germinate unless the products are temperature abused. This aspect has been explained along with microbial growth in the canned products and canning (see Chapter 4 and Chapter 32).

Low-heat-processed uncured meats, such as roasts, are given heat treatment at an internal temperature of 140 to 160°F (60 to 71°C). Generally, the surface of the meats (and thus most of the microorganisms) is exposed to the final temperature for 1 h or more, depending on the size of the meat (which could be more than 10 lb [4.6 Kg]). Under this condition, only the spores of *Bacillus* and *Clostridium* spp. and some extremely thermoduric vegetative species (*Lab. viridescens,* some *Enterococcus, Micrococcus* inside the product) can survive. However, the products, even when cooked in bags and not cut into portions, are opened and handled before final vacuum packaging and refrigerated storage.[1,2,6] Many types of microorganisms can enter as postheat contaminants into the products from equipment, personnel, water, and air. In some situations, spices and other ingredients are added to the products after heating, which, in turn, can be the source of microbial contamination of the products. Some products are sliced before vacuum packaging, which increases the chances of contamination on the surface area of the product from the equipment and environment. Psychrotrophic facultative anaerobic and anaerobic bacteria have been implicated in the spoilage of these products. In vacuum-packaged roast beef,

roast turkey, and roast chicken, heterofermentative *Lactobacillus* spp. and *Leuconostoc* spp., as postheat contaminants, have been involved in spoilage, with the accumulation of large quantities of gas (CO_2) and liquid (due to acid production) inside the bag, without causing much flavor, color, or texture changes. Gas production and purge accumulation in vacuum-packaged roast beef by psychrotrophic *Clostridium* spp., along with off-flavor and color changing from brown to pink to red (after 4 weeks), have been detected. In spoiled sliced roast beef, the brown color of roast beef changes to pink (in 1 week) and the beef has a putrid odor (after 6 weeks). *Proteus* and *Hafnia* spp. are involved in spoilage of this product (Chapter 20).

Low-heat-processed cured meats include a wide variety of products such as franks, bologna, ham, and luncheon meats made from beef, pork, and poultry. Meats are mixed with different types of additives to improve color, texture, flavor, shelf life, and safety. Some of these additives are nitrite, salt, dextrose, phosphate, sorbate, erythorbate, nonfat dry milk, soy proteins, carrageenan, and different types of spices. Some of the reduced-fat products have very low fat (≤ 2%) as compared to products normally with ≥30% fat (some regular franks). Some of the products, especially low-fat products, have pH values as high as 6.8 (compared to pH < 6.0 in other products) due to addition of phosphate and other ingredients and higher moisture. They are cooked at an internal temperature of 150 to 160°F (65 to 71°C). Depending on the size of the products, the surface can be exposed to the final temperature for a longer time than the center. As the products are made from ground or chopped meats, microorganisms are distributed throughout the products (as compared with mainly on the surfaces of a roast or retail cuts, but preformed roasts, steaks, etc., made from chopped meat have microorganisms throughout the products). Thus, the thermodurics surviving cooking are present throughout the products. The products, following cooking, are extensively handled before they are again vacuum packaged or packed with modified atmosphere of CO_2 or $CO_2 + N_2$ or sold unpackaged. Depending on the postheat treatment processing steps (slicing, portioning, and skinning) involved, the products can be contaminated with microorganisms from equipment, personnel, air, and water. Some of the microorganisms establish themselves in the processing environment, especially in places difficult to sanitize (or dead spots). In products such as franks, they contaminate only the surface; but in sliced products, they are distributed over the slices following cutting.

Vacuum-packaged and gas-packaged products, during storage, can be spoiled by psychrotrophic *Lactobacillus* spp. (such as homofermentative *Lab. sake, Lab. curvatus,* heterofermentative *Lab. viridescens*) and *Leuconostoc* spp. (such as *Leu. carnosum, Leu. gelidum,* and *Leu. mesenteroides*). In vacuum-packaged products, the package becomes loose because of growth. The products, depending on bacterial types, show cloudy appearance, large accumulation of gas (CO_2) and liquid, slime formation due to bacterial cells and dextran production by *Leuconostoc* spp. in products containing sucrose (or honey), and slight acidic odor. In some products, growth of *Serratia* spp. (*Serratia liquifacience*) causes amino acid breakdown, which then causes an ammonia-like odor (diaper smell). Low-fat-containing, vacuum-packaged turkey rolls (portions or sliced) develop a pink color after ca. 5 weeks, probably because of growth of some lactic acid bacteria. Under aerobic conditions of storage (unpackaged or permeable-film wrapped), some *Lactobacillus* spp. rapidly

produce H_2O_2, which can oxidize nitrosohemochrome (formed by heating nitrosomyoglobin) to brown metmyoglobin or to oxidized porphyrins, some of which are green in color. Refrigerated luncheon meat can develop brown to yellow spots with fluorescence from the growth of *Enterococcus casseliflavus* (Chapter 20). Yeasts and molds can grow inside if a package, because of even undetectable damage, allows air to leak in.

Unpackaged cooked products that do not have carbohydrates can become putrid from the growth of and protein degradation by proteolytic Gram-negative bacteria. If the products are stored for long times, yeasts and molds can also grow, causing off-flavor, discoloration, and sliminess. Because of growth of H_2O_2-producing lactic acid bacteria, they can have green to gray discolorations.

III. EGGS AND EGG PRODUCTS

A. Shell Eggs

The pores in the eggshell and inner membrane do not prevent entrance of bacteria and hyphae of molds, especially when the pore size increases during storage. The presence of moisture enhances the entrance of motile bacteria. The albumen (egg white) and yolk have ca. 0.5 to 1.0% carbohydrate and are high in proteins but low in nonprotein nitrogen. During storage, the pH can shift to the alkaline side (pH 9 to 10). In addition, lysozyme (causes lysis of bacterial cell wall mucopeptide), conalbumin (chelates iron), antivitamin proteins (avidin binds riboflavin), and protease inhibitors in eggs inhibit microbial growth. The most predominant spoilage of shell eggs is caused by Gram-negative motile rods from several genera that include *Pseudomonas, Proteus, Alcaligenes, Aeromonas*, and the coliform group. The different types of spoilage are designated as rot. Some examples are green rot, causing greening of albumen because of growth of *Pseudomonas fluorescens*; black rot, causing muddy discoloration of yolk because of H_2S production by *Proteus vulgaris*; and red rot by *Ser. mercescens*, caused by production of red pigment. On some occasions, molds from genera *Penicillium, Alternaria,* and *Mucor* can grow inside eggs, especially when the eggs are oiled, and produce different types of fungal rots.[1,2]

B. Egg Products

Liquid eggs consisting of whole, yolk, or white are generally pasteurized or frozen, or both, to prevent microbial growth. If the liquid products are held at room temperature following breaking prior to pasteurization, spoilage bacteria can grow and cause off-flavor (putrid), sourness, or fish flavor (due to formation of trimethylamine). Pasteurized eggs at refrigerated temperature have a limited shelf life unless additional preservatives are added. The predominant bacteria in pasteurized products are some Gram-positive bacteria that survive pasteurization, but spoilage is mainly caused by psychrotrophic Gram-negative bacteria getting into products after heat treatment. Dried eggs are not susceptible to microbial spoilage, because of low A_w.

IV. FISH, CRUSTACEANS, AND MOLLUSKS

A. Fish

Fish harvested from both fresh and saltwater are susceptible to spoilage through autolytic enzyme actions, oxidation of unsaturated fatty acids, and microbial growth. Protein hydrolysis by autolytic enzymes (proteinases) is predominant if the fish are not gutted following catch. Oxidation of unsaturated fatty acids is also high in fatty fish. Microbial spoilage is determined by the microbial types, their level, fish environment, fish types, methods used for harvest, and subsequent handling. These aspects have been discussed in Chapter 4. Fish tissues have high levels of NPN compounds (free amino acids, trimethylamine oxide, and creatinine), peptides, and proteins, but almost no carbohydrates; the pH is generally above 6.0. Gram-negative aerobic rods, such as *Pseudomonas* spp., *Acinetobacter, Moraxella,* and *Flavobacterium*, and facultative anaerobic rods, such as *Shewanella, Alcaligenes, Vibrio,* and coliforms, are the major spoilage bacteria. However, because of the relatively shorter generation time, spoilage by psychrotrophic *Pseudomonas* spp. predominates under aerobic storage at both refrigerated and slightly higher temperature. In fish stored under vacuum or CO_2, lactic acid bacteria (including *Enterococcus*) can become predominant.[1,2]

Gram-negative rods initially metabolize the NPN compounds by decay (oxidation), followed by putrefaction to produce different types of volatile compounds such as NH_3, trimethylamine ($N{:}CH_3$; from reduction of trimethylamine oxide), histamine (from histidine; cause of Scombroid poisoning), putrescine, cadaverine, indoles, H_2S, mercaptans, dimethyl sulfide (especially by *She. putrefaciens*), and volatile fatty acids (acetic, isobutyric, and isovaleric acids). Proteolytic bacterial species also produce extracellular proteinases that hydrolyze fish proteins and supply peptides and amino acids for further metabolism by spoilage bacteria. The volatile compounds produce different types of off-odors, namely, stale, fishy (due to trimethylamine), and putrid. Bacterial growth is also associated with slime production, discoloration of gills and eyes (in whole fish), and loss of muscle texture (soft due to proteolysis).

In fish stored by vacuum or by CO_2 packaging, growth of aerobic spoilage bacteria is prevented. However, anaerobic and facultative anaerobic bacteria can grow, including lactic acid bacteria. Under refrigeration, products have relatively long shelf life due to slower growth of spoilage bacteria. Salted fish, especially lightly salted fish, are susceptible to spoilage by halophilic bacteria such as *Vibrio* (at lower temperature) and *Micrococcus* (at higher temperature). Smoked fish, especially with lower A_w, inhibit growth of most bacteria. However, molds can grow on the surface. Minced fish flesh, surimi, and seafood analogs prepared from fish tissues generally have high initial bacterial levels due to extensive processing (ca. $10^{5–6}$/g). The types include those present in fish and those that get in during processing. These products, such as fresh fish, can be spoiled rapidly by Gram-negative rods unless frozen quickly or used soon after thawing. Canned fish (tuna, salmon, and sardines) are given heat treatment to produce commercially sterile products. They can be spoiled by thermophilic sporeformers unless proper preservation and storage conditions are used.[1–4]

B. Crustaceans

Microbial spoilage of shrimps is more prevalent than that of crabs and lobsters. Whereas crabs and lobsters remain alive until they are processed, shrimps die during harvest. The flesh of crustaceans is rich in NPN compounds (amino acids, especially arginine, trimethylamine oxide), contains ca. 0.5% glycogen, and has a pH above 6.0. The predominant microflora are *Pseudomonas* and several Gram-negative rods. If other necessary factors are present, the nature of spoilage is quite similar to that in fresh fish. Microbial spoilage of shrimp is dominated by odor changes due to production of volatile metabolites of NPN compounds (from decay and putrefaction), slime production, and loss of texture (soft) and color. If the shrimps are processed and frozen rapidly, the spoilage can be minimized. Lobsters are frozen following processing or sold live and thus are not generally exposed to spoilage conditions. Crabs, lobsters, and shrimp are also cooked to extend their shelf life. However, they are subsequently exposed to conditions that cause postheat contamination and then stored at low temperature (refrigerated and frozen). Blue crabs are steamed under pressure, and the meat is picked and marketed as fresh crabmeat. To extend shelf life (and safety), the meat is also heat processed (85°C for 1 min) and stored at refrigerated temperature. Under refrigerated conditions, they have a limited shelf life because of growth of surviving bacteria and postheat contaminants.

C. Mollusks

As compared with fish and crustaceans, oyster, clam, and scallop meats are lower in NPN compounds but higher in carbohydrates (glycogen, 3.5 to 5.5%), with pH normally above 6.0. The mollusks are kept alive until processed (shucked); thus, microbiological spoilage occurs only after processing. The resident microflora are predominantly *Pseudomonas* and several other Gram-negative rods. During refrigerated storage, microorganisms metabolize both NPN compounds and carbohydrates. Carbohydrates can be metabolized to produce organic acids by lactic acid bacteria (*Lactobacillus* spp.), enterococci, and coliforms, thereby lowering the pH. Breakdown of nitrogenous compounds primarily by *Pseudomonas* and *Vibrio*, especially at refrigerated temperature, results in production of NH_3, amines, and volatile fatty acids.

V. MILK AND MILK PRODUCTS

A. Raw Milk

Raw milk contains many types of microorganisms coming from different sources. The average composition of cow's milk is protein 3.2%, carbohydrates 4.8%, lipids 3.9%, and minerals 0.9%. Besides casein and lactalbumin, it has free amino acids, which can provide a good N source (and some C source, if necessary). As the main carbohydrate is lactose, those microorganisms with lactose-hydrolyzing enzymes (phospho-β-galactosidase or β-galactosidase) have an advantage over those unable to metabolize lactose. Milk fat can be hydrolyzed by microbial lipases, with the release of small-molecular volatile fatty acids (butyric, capric, and caproic acids).

Microbial spoilage of raw milk can potentially occur from the metabolism of lactose, proteinaceous compounds, fatty acids (unsaturated), and the hydrolysis of triglycerides. If the milk is refrigerated immediately following milking and stored for days, the spoilage will be predominantly caused by Gram-negative psychrotrophic rods, such as *Pseudomonas, Alcaligenes, Flavobacterium* spp., and some coliforms. *Pseudomonas* and related species, being lactose negative, metabolize proteinaceous compounds to change the normal flavor of milk to bitter, fruity, or unclean. They also produce heat-stable lipases (producing rancid flavor) and heat-stable proteinases that have important implications; this is discussed in Chapter 21. The growth of lactose-positive coliforms produces lactic acid, acetic acid, formic acid, CO_2, and H_2 (by mixed-acid fermentation) and causes curdling, foaming, and souring of milk. Some *Alcaligenes* spp. (*Alc. faecalis*) and coliforms can also cause ropiness (sliminess) by producing viscous exopolysaccharides. However, if the raw milk is not refrigerated soon, growth of mesophiles, such as species of *Lactococcus, Lactobacillus, Enterococcus, Micrococcus, Bacillus, Clostridium,* and coliforms, along with *Pseudomonas, Proteus*, and others, predominates. But lactose-hydrolyzing species, such as *Lactococcus* spp. and *Lactobacillus* spp., generally predominate, producing enough acid to lower the pH considerably and prevent or reduce growth of others. In such situations, curdling of milk and sour flavor is the predominant spoilage. If other microorganisms also grow, gas formation, proteolysis, and lipolysis become evident. Yeast and mold growth, under normal conditions, is generally not expected.[1–3,7]

B. Pasteurized Milk

Raw milk is pasteurized before it is sold for consumption as liquid milk. Thermoduric bacteria (*Micrococcus, Enterococcus,* some *Lactobacillus*, *Streptococcus, Corynebacterium*, and spores of *Bacillus* and *Clostridium*) survive the process. In addition, coliforms, *Pseudomonas, Alcaligenes, Flavobacterium*, and similar types can enter as postpasteurization contaminants. Pasteurized milk, under refrigerated storage, has a limited shelf life, mainly due to growth of these psychrotrophic contaminants. Their spoilage pattern is the same as described for raw milk spoilage. Flavor defects from their growth are detectable when the population reaches $\geq 10^6$ cells/ml. Growth of psychrotrophic *Bacillus* spp., such as *Bacillus cereus*, has been implicated in the spoilage of pasteurized refrigerated milk, especially when the levels of postpasteurization contaminants are low. Spores of psychrotrophic *Bacillus* spp., surviving pasteurization, germinate and multiply to cause a defect known as bitty. They produce the enzyme lecithinase, which hydrolyzes phospholipids of the fat globule membrane, causing aggregation of fat globules that adhere to the container surfaces. Production of rennin-like enzymes by psychrotrophic *Bacillus* spp. and others can cause sweet curdling of milk at a pH higher than that required for acid curdling.[8]

Ultrahigh temperature-treated (UHT) milk (150°C for a few seconds) is an essentially commercially sterile product that can only contain viable spores of some thermophilic bacteria. The UHT milk is not susceptible to spoilage at ambient storage temperature but can be spoiled if exposed to high temperature (40°C or above as observed with canned foods).

C. Concentrated Liquid Products

Evaporated milk, condensed milk, and sweetened condensed milk are the principal types of concentrated dairy products susceptible to limited microbial spoilage during storage. All these products are subjected to sufficient heat treatments to kill vegetative microorganisms as well as spores of molds and some bacteria.

Evaporated milk is condensed whole milk with 7.5% milk fat and 25% total solids. It is packaged in hermetically sealed cans and heated to obtain commercial sterility. Under proper processing conditions, only thermophilic spores of spoilage bacteria can survive, and exposure to high storage temperature (43°C or higher) can trigger their germination and subsequent growth. Under such conditions, *Bacillus* species, such as *Bac. coagulans*, can cause coagulation of milk (flakes, clots, or a solid curd).

Condensed milk is generally condensed whole milk and has 10 to 12% fat and 36% total solids. The milk is initially given a low-heat treatment, close to pasteurization temperature, and then subjected to evaporation under partial vacuum (at ca. 50°C). Thus, it can have thermoduric microorganisms that subsequently can grow and cause spoilage. Other microorganisms can also get into the product during the condensing process. Even at refrigerated temperature, this product has a limited shelf life, as does pasteurized milk.

Sweetened condensed milk contains ca. 8.5% fat, 28% total solids, and 42% sucrose. The whole milk is initially heated to a high temperature (80 to 100°C) and then condensed at ca. 60°C under vacuum and put into containers. Because of a low A_w, it is susceptible to spoilage from the growth of osmophilic yeasts (such as *Torula* spp.), causing gas formation. If the containers have enough headspace and oxygen, molds (e.g., *Penicillium* and *Aspergillus*) can grow on the surface.

D. Butter

Butter contains 80% milk fat and can be salted or unsalted. The microbiological quality of butter depends on the quality of cream and the sanitary conditions used in the processing. Growth of bacteria (*Pseudomonas* spp.), yeasts (*Candida* spp.), and molds (*Geotrichum candidum*) on the surface causes flavor defects (putrid, rancid, or fishy) and surface discoloration. In unsalted butter, coliforms, *Enterococcus*, and *Pseudomonas* can grow favorably in the water phase (which has nutrients from milk) and produce flavor defects.

VI. VEGETABLES AND FRUITS

A. Vegetables

Fresh vegetables contain microorganisms coming from soil, water, air, and other environmental sources, and can include some plant pathogens. They are fairly rich in carbohydrates (5% or more), low in proteins (ca. 1 to 2%), and, except for tomatoes, have high pH. Microorganisms grow more rapidly in damaged or cut

vegetables. The presence of air, high humidity, and higher temperature during storage increases the chances of spoilage. The most common spoilage is caused by different types of molds, some of those from genera *Penicillium, Phytopthora, Alternaria, Botrytis,* and *Aspergillus.* Among the bacterial genera, species from *Pseudomonas, Erwinia, Bacillus*, and *Clostridium* are important.[1–3]

Microbial vegetable spoilage is generally described by a common term *rot*, along with the changes in the appearance, such as black rot, gray rot, pink rot, soft rot, stem-end rot. In addition to changes in color, microbial rot causes off-odor and loss of texture. This is more apparent in ready-to-eat cut salads, and sliced vegetables and fruits that are refrigerated for a long time (3 to 4 weeks) and can be temperature abused.

Refrigeration, vacuum or modified atmosphere packaging, freezing, drying, heat treatment, and chemical preservatives are used to reduce microbial spoilage of vegetables. Spoilage of canned vegetables, vegetable juices, and fermented vegetables are discussed later in this chapter.

B. Fruits

Fresh fruits are high in carbohydrates (generally 10% or more), very low in proteins (≤1.0%), and have a pH 4.5 or below. Thus, microbial spoilage of fruits and fruit products is confined to molds, yeasts, and aciduric bacteria (lactic acid bacteria, *Acetobacter, Gluconobactor*). Like fresh vegetables, fresh fruits are susceptible to rot by different types of molds from genera *Penicillium, Aspergillus, Alternaria, Botrytis, Rhizopus*, and others. According to the changes in appearance, the mold spoilage is designated as black rot, gray rot, soft rot, brown rot, or others. Yeasts from genera *Saccharomyces, Candida, Torulopsis*, and *Hansenula* are associated with fermentation of some fruits, such as apples, strawberries, citrus fruits, and dates. Bacterial spoilage associated with souring of berries and figs has been attributed to the growth of lactic acid and acetic acid bacteria.[1–3]

To reduce spoilage, fruits and fruit products are preserved by refrigeration, freezing, drying, reducing A_w, and heat treatment. Spoilage of fruit juices, jams, and wine is discussed later.

VII. SOFT DRINKS, FRUIT JUICES AND PRESERVES, AND VEGETABLE JUICES

Carbonated and noncarbonated soft drinks, fruit juices, and preserved and concentrated fruit juices and drinks are low-pH products (pH 2.5 to 4.0). The carbohydrate (sucrose, glucose, and fructose) content ranges from 5 to 15% in juices and drinks, but 40 to 60% in concentrates and preserves. High sugar content reduces the A_w of these products, which in the concentrates and preserves can be ca. 0.9. Carbonated beverages also have low O–R potential.

Among the microorganisms that can be present in these products, only aciduric molds, yeasts, and bacteria (*Lactobacillus, Leuconostoc*, and *Acetobacter*) are able to cause spoilage if appropriate preservation methods are not used. In carbonated

beverages, some yeast species from genera *Torulopsis, Candida, Pichia, Hansenula,* and *Saccharomyces* can grow and make the products turbid. Some *Lactobacillus* and *Leuconostoc* species can also grow to cause cloudiness and *Leuconostoc* can cause ropiness (due to production of dextrans) of the products. Noncarbonated beverages can be similarly spoiled by yeasts, *Lactobacillus*, and *Leuconostoc* spp. In addition, if there is enough dissolved oxygen, molds (*Penicillium, Aspergillus, Mucor,* and *Fusarium*) and *Acetobacter* can grow; the latter produces acetic acid to give a vinegar-like flavor. Fruit juices are susceptible to spoilage by molds, yeasts, *Lactobacillus, Leuconostoc*, and *Acetobacter* spp. However, a particular type of juice may be susceptible to spoilage by one or another type of microorganism. Molds and *Acetobacter* can grow if enough dissolved oxygen is available. Yeasts can cause both oxidation (producing CO_2 and H_2O) and fermentation (producing alcohol and CO_2) of the products. *Acetobacter* can use alcohol to produce acetic acid. Heterofermentative *Lab. fermentum* and *Leu. mesenteroides* can ferment carbohydrates to lactate, ethanol, acetate, CO_2, diacetyl, and acetoin. In addition, *Leu. mesenteroides* and some strains of *Lab. plantarum* can produce slime due to production of dextran and other exopolysaccharides. In fruit drinks, *Lactobacillus* and *Leuconostoc* spp. can also convert citric and malic acids (additives) to lactic and acetic acids and reduce the sour taste (flat flavor). Because concentrated fruit drinks and fruit preserves have a low A_w (0.9), only osmophilic yeasts can grow; molds also can grow if oxygen is available. A new group of bacterial species, *Alicyclobacillus* (e.g., *Alicyclobacillus acidoterrestris*), has been recognized recently to cause spoilage of low-pH fruits and vegetable juices.

To prevent growth of these potential spoilage microorganisms, several additional preservation methods are used for these products, which include heat treatment, freezing, refrigeration, and addition of specific chemical preservatives.

Tomato juice has a pH of ca. 4.3. It is generally given high heat treatment to kill vegetative microorganisms. However, bacterial spores can survive. Flat sour spoilage of tomato juice due to germination and growth of *Bac. coagulans* is documented. Most other vegetable juices have pH values between 5.0 and 5.8, and many have growth factors for lactic acid bacteria. These products are susceptible to spoilage because of the growth of many types of microorganisms. Effective preservation methods are used to control their growth.

VIII. CEREALS AND THEIR PRODUCTS

Some of the products susceptible to microbial spoilage include high-moisture cereal grains, germinated or sprouted seeds and grains, refrigerated dough, breads, soft pastas, and pastries.[1,2]

A. Grains and Seeds

Grains and seeds normally have 10 to 12% moisture, which lowers the A_w to ca. ≤0.6 and thus inhibits microbial growth. In cereal grains, during harvesting, processing, and storage, if the A_w increases above 0.6, some molds can grow. Some

species of storage fungi from genera *Aspergillus*, *Penicillium,* and *Rhizopus* can cause spoilage of high-moisture grains. Many types of lentils, beans, and seeds (alfalfa) are germinated and used as ready-to-eat products. During storage, Gram-positive and Gram-negative bacteria, yeasts, and molds can grow and produce off-flavor.

B. Refrigerated Dough

Refrigerated dough (for biscuits, rolls, and pizza) is susceptible to spoilage (gas formation) from the growth of psychrotrophic heterolactic acid bacterial species of *Lactobacillus* and *Leuconostoc*. Rapid CO_2 production can blow the containers, especially when the storage temperature increases to 10°C and above.

C. Breads

The A_w of breads is normally low enough (0.75 to 0.9) to prevent growth of bacteria. However, some molds (bread molds: *Rhizopus stolonifer*) can grow, especially if moisture is released because of starch crystallization during storage. Molds are killed during baking; however, spores can get in from air and equipment following baking. When breads are frozen, they may contain ice crystals in the bags. Following thawing, some portions can absorb enough moisture for yeasts and bacteria to grow and cause spoilage (sour taste, off-flavor). A specific type of bread spoilage, designated as ropiness and characterized by a soft, stringy, brown mass with fruity odor, is caused by the growth of some mucoid variants of *Bac. subtilis*. The spores, coming from flour or equipment, survive baking and then germinate and grow inside within 1 to 2 d. They also produce extracellular amylases and proteases and break down the bread structure. High moisture inside the bread, slow cooling, and pH above 5.0 favor ropiness.

D. Pastas

Pastas can be spoiled by microorganisms before drying because of improper manufacturing practices. Dry pastas do not favor microbial growth. However, soft pastas can be spoiled by bacteria, yeasts, and molds. Anaerobic packing and refrigeration storage can prevent mold growth and slow down growth of yeasts and psychrotrophic anaerobic and facultative anaerobic bacteria. Suitable preservatives can be used to prevent their growth.

E. Pastries

Pastries include cakes and baked shells filled with custard, cream, or sauces. They can be spoiled by microorganisms coming with the ingredients that are added after baking, such as icing, nuts, toppings, and cream. Most products, because of low A_w, allow only molds to grow. However, some materials used as fillings may have high A_w, which allows for bacterial growth.

IX. LIQUID SWEETENERS AND CONFECTIONERIES

Liquid sweeteners include honey, sugar syrups, maple syrups, corn syrups, and molasses. Confectionery products include soft-centered fondant, cream, jellies, chocolate, and Turkish delight. Most of these products have an A_w of 0.8 or below, and are normally not susceptible to bacterial spoilage. Under aerobic conditions, some xerophilic molds can produce visible spoilage. However, osmophilic yeasts from genera *Zygosaccharomyces (Zygosaccharomyces rouxii), Saccharomyces (Saccharomyces cerevisiae), Torulopsis (Torulopsis holmii),* and *Candida (Candida valida)* can ferment these products. To prevent growth of yeasts in some of these products with slightly higher A_w (such as in maple syrup), chemical preservatives are added.

X. MAYONNAISE, SALAD DRESSINGS, AND CONDIMENTS

These products normally contain some molds, yeasts, spores of *Bacillus* and *Clostridium*, and aciduric bacteria such as *Lactobacillus* and related species. Because of low pH, acid-sensitive bacteria may not survive long. Mayonnaise, with 65% or more edible oil and ca. 0.5% acetic acid, has an A_w of ca. 0.92 and a pH of 3.6 to 4.0. Salad dressings generally contain 30% or more edible oil, 0.9 to 1.2% acetic acid, an A_w of 0.92, and a pH of 3.2 to 3.9. The major factors controlling microbial growth are undissociated acetic acid, low pH, and relatively low A_w. However, some aciduric microorganisms can grow to cause spoilage. Molds can grow only on the surface exposed to air. Some microaerophilic and facultative anaerobic yeasts and heterolactic *Lactobacillus* spp. (especially those that can grow at an A_w of 0.92) can also multiply to produce CO_2. *Lab. fructivorans* hydrolyzes sucrose present in the products and produces gas (CO_2), especially from rapid metabolism of fructose that is released from sucrose. Strains of *Sac. bailii* have also been implicated in gas spoilage (CO_2 and alcohol) of these products. *Lab. fructivorans* cells usually die rapidly after multiplication and are difficult to isolate unless specific methods are used. This can lead to a wrong assumption regarding the microorganisms that cause spoilage of these products.

Low-calorie salad dressing, in which oil and acetic acid are added in much lower concentrations, has high pH and A_w values. Many microorganisms can grow in these products. To enhance their shelf life (and safety), refrigerated storage is recommended.

Yeasts, *Lactobacillus* spp., and *Leuconostoc* spp. can also cause gassy spoilage of catsup, salsa, sauces, and prepared mustard. Some *Bacillus* spp. have also been implicated in gassy spoilage of mustard preparations. To control growth of spoilage microorganisms, additional preservation methods, especially chemical preservatives, are used.[1,2,9]

XI. FERMENTED FOODS

Desirable microorganisms are used directly or indirectly to produce many types of fermented foods and beverages from meat, fish, milk, vegetables, fruits, cereal grains,

and others (Chapter 14). The desirable microorganisms are present in very high numbers and the products contain either high levels of organic acids or alcohol. In addition, the products have a low pH and some have a low A_w (e.g., dry salami). Generally, these products have a longer shelf life. However, under certain conditions, they are susceptible to microbial spoilage. This aspect is discussed briefly.[1,2]

A. Fermented Meat Products

Fermented meat products normally have a pH between 4.5 and 5.0 and an A_w between 0.73 and 0.93 (Chapter 14). During fermentation, if the acid production of homofermentative lactic acid bacteria is slow, undesirable bacteria can grow. *Clostridium, Bacillus*, and other mesophilic bacteria have been reported to cause spoilage in such conditions. Products with a pH lower than 5.0 but an A_w of 0.92 or above and vacuum packaged can be spoiled by heterofermentative *Leuconostoc* and *Lactobacillus* spp., with the accumulation of gas and liquid inside the package and creamy white growth of the bacterial cells. If they are not vacuum packaged and have a low A_w (0.72 to 0.90), yeasts and molds can grow on the surface, resulting in slime formation, discoloration, and undesirable flavor of the products.

B. Fermented Dairy Products

Cultured buttermilk, yogurt, and cheeses are a few of the large number of fermented dairy products generally produced by inoculating milk with specific starter-culture bacteria (Chapter 14). They differ in their acidity, A_w, and storage stability. Buttermilk generally has ca. 0.8% lactic acid and a pH of 4.8. Yeasts can grow and cause spoilage by producing gas. Some strains of starter cultures can produce exopolysaccharides to give a slimy texture (which is desirable in some products).

Plain yogurt generally has a pH of 4.5 or lower (with ca. 1% lactic acid) and is not spoiled by undesirable bacteria. However, the product can develop a bitter flavor due to production of bitter peptides by some strains of *Lab. delbrueckii* ssp. *bulgaricus* used as a starter culture. During storage, the starter bacteria can continue to produce lactic acid, causing an objectionable sharp acid taste. Both starters can produce exopolysaccharides, causing sliminess of the product. Yeasts (especially in fruit yogurts) may grow in the acid environment and produce CO_2 as well as yeasty and fruity off-flavors. In blended yogurts, many of these changes are not detected because of added ingredients. Some species of molds can grow on the surface if yogurt is stored for a long time.

Microbial cheese spoilage is greatly influenced by A_w and pH. Unripened cottage cheese with high moisture content and low acidity is susceptible to spoilage by Gram-negative bacteria, predominantly psychrotrophic rods, and yeasts and molds. *Alcaligenes* and *Pseudomonas* spp. are frequently involved, causing a slimy texture and an unclean, putrid flavor. Some unripened ethnic cheeses (such as Mexican-style) are vacuum packaged and stored at refrigerated temperature for a shelf life of ca. 30 d or more (see Chapter 20). They are occasionally spoiled by heterofermentative *Leuconostoc* spp., characterized by gas (CO_2) and liquid accumulation in the bag. Gassiness in some cheeses with high pH, low salt, and relatively high A_w

(such as Gouda, Emmentaler, and provolone) can also occur from the growth of some *Clostridium* (e.g., *Clo. tyrobutyricum*). Their spores survive pasteurization of milk, germinate, and grow in the anaerobic environment to produce CO_2, H_2, and butyrate from the metabolism of lactate. Hard-ripened cheese, such as cheddar, can have a bitter taste due to rapid production of bitter peptides during ripening. Fast acid-producing strains of *Lactococcus lactis* used as starters are generally associated with this defect. Sharp-ripened cheeses can also have large amounts of biologically active amines (e.g., histamine and tyramine), produced from the decarboxylation of the respective amino acids by decarboxylase. Decarboxylase enzymes can be present in some starter strains or in secondary microflora of the cheeses (*Enterococcus,* some coliforms). Lysis of the cells releases the enzymes during the ripening process, causing decarboxylation of amino acids and accumulation of these amines. Hard and semihard cheeses are generally susceptible to spoilage from mold growth on the surface and produce undesirable color and flavor defects in the products. Anaerobic packaging greatly reduces this problem.

C. Fermented Vegetable and Fruit Products

Many types of vegetables are fermented, among which cucumber and sauerkraut are produced in large volumes. In salt stock pickles containing ca. 15% salt, yeasts and halophilic bacteria can grow, especially if the acidity is not sufficient. Dill pickles with low salt (<5%) can have a bloating defect from CO_2 production by yeasts, heterofermentative lactic acid bacteria, and coliforms, especially if the desirable bacteria associated with fermentation do not grow properly. *Candida, Torulopsis,* and *Saccharomyces* spp. are often the causative yeasts. Sweet and sour pickles preserved with sugar and vinegar can be spoiled from the growth of yeasts and lactic acid bacteria, especially when the acid level is not sufficient. Sauerkraut can be spoiled from the growth of yeasts and molds if the air is not excluded during fermentation of cabbage (see Chapter 14). Failure of lactic acid bacteria to grow rapidly and in proper sequence can lead to low acid production. Under this condition, coliforms and other Gram-negative bacteria can multiply to produce undesirable flavor, texture, and color defects. Olives are fermented for a long time and are susceptible to many types of spoilage. The most common problem is gassiness (bloating) due to CO_2 production by heterofermentative lactic acid bacteria, coliforms, and yeasts. Softening of texture can be caused by pectinases of yeasts (*Rhodotorula* spp.).

D. Fermented Beverages

Wines can support growth of film yeasts and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) under aerobic conditions. Film yeasts oxidize alcohol and organic acids and form a surface pellicle, whereas acetic acid bacteria oxidize alcohol to acetic acid and CO_2. Under anaerobic conditions, several lactic acid bacteria (e.g., *Lactobacillus, Leuconostoc,* and *Pediococcus* spp.) can grow in wine. Some heterofermentative *Lactobacillus* spp. can ferment glucose and fructose and increase the acidity of wine, producing a defect called tourne spoilage (by *Lab. brevis, Lab.*

buchneri). They can also produce cloudiness and mousy odor. Some *Leuconostoc* spp. can produce sliminess and cloudiness. *Oenococcus oenos* can convert malic acid to lactic acid and CO_2 and reduce the acidity of a wine. Sometimes, this malolactic fermentation is used advantageously to reduce the sourness of wine.

Beer spoilage can be caused by some lactic acid bacteria and yeasts. Growth of *Pediococcus* spp. causes an increase in acidity and cloudiness. Some *Lactobacillus* spp. can also multiply and cause turbidity. *Acetobacter* and *Gluconobacter*, in the presence of air, can produce cloudiness and sliminess as well as make the beer sour. Wild yeasts (yeasts other than those used in fermentation) can grow in beer, causing off-flavor.

XII. CANNED FOODS

Canned foods are heat treated to kill microorganisms present, and the extent of heat treatment is predominantly dependent on the pH of a food (Chapter 4). High-pH (pH 4.6 and above; also called low-acid) foods are heated to destroy most heat-resistant spores of the pathogenic bacteria, *Clo. botulinum*, to ensure that a product is free of any pathogen.[1,2,10] However, spores of some spoilage bacteria, which have greater heat resistance than do spores of *Clo. botulinum*, can survive. Thus, these products are called commercially sterile (instead of sterile, which means free of any living organism) foods. The spores that survive the heat treatment designed to destroy *Clo. botulinum* spores are thermophilic spores and can germinate at 43°C and above. However, once germinated, some can outgrow at temperatures as low as 30°C. The other food group, designated as low-pH or high-acid food with pH below 4.6, is heat treated to kill all vegetative cells and some spores. Although low pH inhibits germination of spores and subsequent growth of *Clo. botulinum*, spores of some aciduric thermophilic spoilage bacteria can germinate and grow when the products are stored at higher temperatures, even for a short time, which induces germination. Some spores of thermoduric mesophilic spoilage bacteria (including pathogenic) can also survive heating in these products, but they are inhibited to germinate by the low pH.

Canned food spoilage is both due to nonmicrobial (chemical and enzymatic reactions) and microbial reasons. Production of hydrogen (hydrogen swell), CO_2, browning, corrosion of cans due to chemical reactions, and liquification, gelation, and discoloration due to enzymatic reactions are some examples of nonmicrobial spoilage. Microbial spoilage is due to three main reasons: (1) inadequate cooling after heating or high-temperature storage, allowing germination and growth of thermophilic sporeformers; (2) inadequate heating, resulting in survival and growth of mesophilic microorganisms (vegetative cells and spores); and (3) leakage (can be microscopic) in the cans, allowing microbial contamination from outside following heat treatment and their growth.

A. Thermophilic Sporeformers

Thermophilic sporeformers can cause three types of spoilage of low-acid (high-pH) foods (such as corn, beans, peas) when the cans are temperature abused at 43°C and above, even for a short duration.

1. Flat Sour Spoilage

The cans do not swell but the products become acidic because of germination and growth of facultative anaerobic *Bac. stearothermophilus*. Germination occurs at high temperature (43°C and above), but growth can take place at lower temperature (30°C and above). The organism ferments carbohydrates to produce acids without gas but with some off-flavor and cloudiness.

2. Thermophilic Anaerobe (TA) Spoilage

The spoilage is caused by the growth of anaerobic *Clo. thermosaccharolyticum* with the production of large quantities of H_2 and CO_2 gas and swelling of cans and with sour and cheesy odor. Following germination in the thermophilic range (43°C and above), the cells can grow at lower temperatures (30°C and above).

3. Sulfide Stinker Spoilage

This spoilage is caused by the Gram-negative anaerobic sporeformer *Desulfotomaculum nigrificans*. The spoilage is characterized by a flat container but darkened products with the odor of rotten eggs due to H_2S produced by the bacterium. H_2S, produced from the sulfur-containing amino acids, dissolves in the liquid and reacts with iron to form black color of iron sulfide. Both germination and growth occur at thermophilic range (43°C and above).

B. Spoilage Due to Insufficient Heating

Insufficient heat treatment results in the survival of mainly spores of *Clostridium* and some *Bacillus* spp. Following processing, they can germinate and grow to cause spoilage. The most important concern is the growth of *Clo. botulinum* and production of toxins.

Spoilage can be either from the breakdown of carbohydrates or proteins. Several *Clostridium* spp., *Clo. butyricum* and *Clo. pasteurianum*, ferment carbohydrates to produce volatile acids and H_2 and CO_2 gas, causing swelling of cans. Proteolytic species, *Clo. sporogenes* and *Clo. putrefacience* (also proteolytic *Clo. botulinum*), metabolize proteins and produce foul-smelling H_2S, mercaptans, indole, skatole, ammonia, as well as CO_2 and H_2 (causing swelling of cans).

Spores of aerobic *Bacillus* spp., surviving inadequate heating, do not grow in cans. However, spores of some facultative anaerobic *Bacillus* spp., such as *Bac. subtilis* and *Bac. coagulans*, grow, with the production of acid and gas (CO_2).

C. Spoilage Due to Container Leakage

Damaged and leaky containers allow different types of microorganisms to get inside from the environment after heating. They can grow in the food and cause different types of spoilage, depending on the microbial types. Contamination with pathogens makes the product unsafe.

XIII. CONCLUSION

This chapter discusses why different food types or food groups differ in microbial spoilage characteristics. The predominant nutrients being metabolized and the predominant microorganisms growing rapidly under an environment determine the nature of the end products that are produced, which in turn are associated with spoilage characteristics. An understanding of these parameters is important in the firsthand determination of the causes of spoilage. This will be useful to investigate the cause, identify it, and take necessary corrective measures. These aspects are discussed further in Chapter 20, using some real situations in refrigerated food products.

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QUESTIONS

1. List the important psychrotrophic spoilage bacteria of raw meats, and describe the metabolic pattern and corresponding spoilage associated with each microbial genus.
2. Briefly discuss the following: (a) sources of microorganisms in low-heat-processed meat products; (b) predominant spoilage bacteria in low-heat-processed, vacuum-

packaged meat products stored at ≤ 4°C; and (c) factors associated with greening of unpackaged and packaged meat and meat products during refrigerated storage.

3. List four microbial inhibitors in shell eggs and predominant spoilage microflora of eggs and egg products.
4. Briefly explain the following: (a) metabolism of nutrients by predominant spoilage bacteria while growing in fish tissues; (b) postheat contamination and spoilage of crabmeat; and (c) metabolism of nutrients by spoilage bacteria in shucked oyster.
5. List the predominant microflora of pasteurized milk and their sources. Discuss the reasons for the limited shelf life of pasteurized milk.
6. Discuss the predisposing factors associated with microbial spoilage of UHP-treated milk, sweetened condensed milk, and butter.
7. List bacteria, yeasts, and molds (two of each) associated with the spoilage of vegetables and fruits. Discuss the major differences in spoilage by bacteria of these two groups of food. How are ready-to-eat vegetables, fruits, and germinated seeds and beans spoiled by bacteria?
8. Discuss the influence of intrinsic factors associated with microbial spoilage of fruit drinks, fruit juices, and vegetable juices, and the nature of spoilage of these products caused by the predominant microflora.
9. Jams, jellies, peanut butter and salsa are not spoiled at room temperature as long as the bottles/jars are not opened. The labels on the product, however, suggest to refrigerate the products after opening. What could be the reasons?
10. List the similarities and differences in the microbial spoilage of (a) breads, (b) pastries, (c) soft pastas, and (d) confectioneries.
11. List the microorganisms involved and the predisposing causes for the following spoilage conditions: (a) gassy defect in mayonnaise, (b) gassy defect of prepared mustard, (c) gassy defect in unripened soft cheese (such as Mexican-style cheese) and Gouda cheese, (d) bitter taste in yogurt and cheddar cheese, (e) tourne spoilage of wine, (f) malolactic fermentation in wine, and (g) sour beer.
12. List the major causes of spoilage of canned foods. Describe the factors, causative bacterial species, and the nature of spoilage of canned foods produced by thermophilic sporeformers.

CHAPTER 20

New Food Spoilage Bacteria in Refrigerated Foods

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I. MICROORGANISMS THAT GROW IN REFRIGERATED FOODS (PSYCHROTROPHS)

In food microbiology, the terms *psychrophiles* and *psychrotrophs* are used to identify those microorganisms that can grow in food stored at low temperatures, namely, chilling and refrigeration, that can range between –1°C and +7°C. A present-day domestic refrigerator is expected to maintain a temperature of ca. 40°F (4.4°C), whereas commercial refrigeration can be lower or higher than this, depending on the particular food stored and shelf life expected. Highly perishable foods are either refrigerated or chilled on ice. Thus, psychrotrophs can multiply in these foods, provided other conditions for growth are not restricting.[1,2]

There is some confusion about the definitions of the two terms, particularly in relation to their importance in food microbiology. The term *psychrophile* or *psychrophilic* is quite specific and includes those microorganisms that grow optimally at ca. 12 to 15°C and have a growth temperature range between ≤ –5°C and 22°C. These microorganisms can grow in refrigerated and chilled foods. The definition of *psychrotrophs* is, however, not clear-cut. Originally, in 1960, this term was introduced to include those microorganisms that grow at 0 to 5°C, irrespective of their optimum or the range of growth temperatures. Rather, they seem to grow best at 25 to 30°C and might not grow above 35°C. Thus, they appear to be a subgroup of mesophiles (growth temperature of mesophiles: optimum 30 to 40°C and range 5 to 45°C), but not a subgroup of psychrophiles. This group includes Gram-positive and Gram-negative aerobic, anaerobic, and facultative anaerobic, motile and nonmotile, sporeformers and nonsporeformers, coccus and rod-shaped bacteria, as well as many yeasts and molds. Later in 1976, the International Dairy Foundation defined psychrotrophs as those microorganisms that grow at 7°C, irrespective of their optimal and range of growth temperatures. However, this does not indicate whether it includes only the subgroup of mesophiles with the ability to grow at or below 7°C, or both psychrophiles and the subgroup of mesophiles that grow at the lower temperature range. More recently, the term *psychrotrophs* includes mesophilic subgroups that can grow at 40°F or below (≤ 4.4°C, refrigerated temperature).[1,2]

Several studies have revealed that there are some mesophilic pathogens, (e.g., *Yersinia enterocolitica* and *Listeria monocytogenes*) and spoilage bacteria (e.g., *Leuconostoc* spp., several *Lactobacillus* spp., and *Serratia* spp.), that can grow in vacuum- and modified-air (MA) packaged foods at 0 to 1°C. Spoilage of vacuum-packaged meats by the psychrophilic *Clostridium* spp. with a growth range between –2 and 20°C was also reported. It is important to have understandable communication among the people associated with food production, regulations, sanitation, academic and research activities, and others without creating confusion by using the designations *psychrotrophic spoilage* for the mesophilic subgroup and *psychrophilic spoilage* for the second group. For easier understanding, it is probably not unscientific to use the term *psychrotrophs* for the microorganism that can grow (in food) at refrigerated or chilled storage temperature (≤ 40°F or 4.4°C). This includes both the mesophile subgroup that can grow at lower temperature and psychrophiles. As both groups are important in spoilage and foodborne diseases, and the methods used to

detect and control them do not differentiate the two groups, a single terminology can be used for both. The same system has been used in food microbiology for the term *thermoduric*, the microorganisms that survive pasteurization (or low-heat treatment), and can include thermophiles, mesophiles, and psychrophiles (*Clostridium laramie*). In this book, the term *psychrotroph* is used to include microorganisms that can grow in food stored at refrigeration temperature irrespective of their being psychrophilic or mesophilic.[1,2]

II. POPULARITY OF REFRIGERATED FOODS

The demand for refrigerated foods has increased dramatically in countries where refrigeration systems are economically available for the food processors, retailers, and consumers.[3,4] Three major factors can be attributed to this increase. The first is the need and desire of consumers for convenient foods. The changes in socioeconomic pattern, such as increase in two-income families, single-parent households, singles, the elderly, and college students living away from home, have created a demand for convenient foods. Second, there is an increase in awareness and belief that harshly processed and harshly preserved foods, as well as foods with high fat, cholesterol, and sodium, are not beneficial for a long and healthy life. Health-conscious consumers are interested in foods that are natural and fresh. These two factors have created a market for healthy foods that can be of high quality (restaurant quality), convenient (e.g., take the least time to prepare at home), and economically affordable. Finally, the technologies necessary for economical production of such foods and subsequent handling until they are consumed are available, especially in developed countries. As a result, different types of refrigerated foods, including new-generation refrigerated and chilled foods, sous vide foods, many of which are ready-to-eat, "heat-n-eat," and "microwave-n-eat" types, are being commercially produced. Many of the new-generation foods are given low-heat treatment to no heat treatment, contain little or no preservatives, and many are vacuum-packaged or packaged with modified atmosphere (MA); with 100% CO_2 or a mixture of CO_2 and nitrogen without or with some oxygen). They are expected to have a shelf life of 20 to 60 d or more. Vacuum- or MA-packaged refrigerated unprocessed or raw foods are also expected to have a much longer shelf life than the same foods stored in the presence of air. Sous vide foods are fresh foods (e.g., vegetables, meat, or fish) that are vacuum packaged, cooked at low heat, and stored in a refrigerator or over ice. They are warmed in the package before serving and eating, and are expected to have a 3-week shelf life. Fresh and ready-to-eat cut fruits and vegetables are packaged with air and have a shelf life of ca. 2 weeks at refrigerated or higher temperatures.

Consumers prefer refrigerated foods because of their better taste, texture, quality, and convenience. They also perceive these foods to be relatively fresh, nutritious, and close to natural, as compared with frozen, canned, or dried foods and fast foods. The popularity of these refrigerated or chilled foods has convinced food experts that this is not a fad — it is here to stay and the demand will continue to increase in the future.[3,4]

III. MICROBIOLOGICAL PROBLEMS

Vacuum- or MA-packaged refrigerated (≤5°C) foods are expected to inhibit the growth of aerobic, most mesophilic, and thermophilic microorganisms. However, if the product contains dissolved or trapped oxygen and the modified air contains oxygen, aerobic and microaerophilic microorganisms capable of growing at refrigerated temperatures can also grow as long as the oxygen is available. Similar possibilities also exist if the packaging material is relatively permeable to atmospheric oxygen.[5]

Even under ideal conditions (no oxygen and refrigeration at ≤5°C), the vacuum-packaged unheated foods normally harbor both anaerobic and facultative anaerobic spoilage and pathogenic bacteria that can multiply during refrigerated storage. Heat-processed products are usually given a low-heat treatment that, depending on a product, varies from 60 to 74°C (140 to 170°F) for a specified time. At this temperature, pathogenic and spoilage bacterial spores and cells of some thermoduric bacteria can survive. Some of these survivors can be anaerobic and facultative anaerobic and can multiply at refrigerated temperatures. Many of these heat-processed products are, however, handled extensively following heating and before final repackaging under vacuum or MA. Pathogenic and spoilage bacteria that can grow during storage can also get into the products during this time as postheat contaminants. Thus, these products have the potential of harboring pathogens and spoilage bacteria that can multiply under vacuum or MA at refrigerated temperature. As these products are expected to have a long shelf life, some up to 100 d, even a very low population ($\leq 10^1$/g) of psychrotrophic anaerobic and facultative anaerobic bacteria can multiply and reach a level that causes spoilage of the food or makes it unsafe. These products are often temperature abused during transportation, in display cases at the retail store, and at homes of consumers. Depending on the time and temperature of abuse, the product temperature can go to 10 to 15°C for a considerable period. The shelf life and safety of the products can be drastically reduced because of accelerated growth rates of psychrotrophs, as well as by some mesophiles (anaerobic and facultative anaerobic) that do not grow at ≤ 5°C but can at the abusive temperature. It has been suspected that even a few hours (4 to 6 h) at 12 to 15°C can reduce the shelf life of such products by 8 to 10 d.

There is concern among regulatory agencies regarding the safety of these products, because the incidences of foodborne diseases following consumption of pasteurized refrigerated foods have been recorded (e.g., *Lis. monocytogenes* in ready-to-eat processed meat products). Food processors are also encountering spoilage of these products in higher frequencies (see examples later). To overcome the microbiological problems in these foods, the National Food Processors Association has recommended several guidelines. These include selecting good-quality raw materials, installing good sanitary procedures, incorporating hazard analysis critical control points (HACCP) at all phases between production and consumption of these foods (from field to table), and, where possible (Chapter 32), applying the highest permissible heat treatment to a product at the final step and

eliminating or minimizing postheat treatment contamination. Other recommendations are to incorporate multiple barriers or hurdles (Chapter 40) along with refrigeration and vacuum or MA packaging. Some of these can be incorporated into the product formulation to reduce the pH and A_w. In addition, acceptable and suitable preservatives (e.g., biopreservatives; see Chapter 16) can be included to combat microbial survival and growth. Some packaged products, such as processed meats, can also be given a hydrostatic pressure processing treatment (Chapter 39).

Many of the food processors are following one or more of these recommendations, especially good sanitation. This helps reduce the initial microbial population of the products considerably. Yet incidences of spoilage of (and foodborne diseases from) vacuum- or MA-packaged refrigerated products are not infrequent and, at times with some processors, spoilage occurs at epidemic proportion both in raw and low-heat-processed products. Many of the bacteria frequently isolated from such spoiled products are being recognized either as new species or species that were not of major concern before. Some of these are *Clo. laramie, Clo. estertheticum, Clo. algidicarnis, Carnobacterium* spp., *Leuconostoc carnosum, Leu. gelidum, Lactobacillus sake, Lab. curvatus,* atypical or unidentifiable lactobacilli and leuconostocs, *Brochothrix thermosphacta, Enterococcus* spp., *Serratia liquifaciens, Hafnia* spp., *Proteus* spp., and some other *Enterobacteriaceae*. Except for spores of *Clostridium* spp., and some thermodurics (some lactobacilli and enterococci), cells of the others are sensitive to heat treatment (pasteurization) given to the processed products. Thus, they are getting in heated products as postheat contaminants on the product surfaces (so are some pathogens). There is a growing speculation and belief among scientists that although these bacterial species have become major causes of spoilage of vacuum- or MA-packaged refrigerated foods, most are neither new nor variants of the existing species. They are present in the environment but probably as minor flora. Three reasons could have helped them become major spoilage organisms. The first could be the changes in the environment of food, such as efficient vacuum (or compositions used in MA) packaging and oxygen barrier systems, higher pH in some meat products (low fat, high phosphate), and long storage time at low temperature; these might have controlled the growth of traditional bacteria associated with food spoilage before but are not efficient for these nontraditional bacteria. A second reason could be that in order to produce a safer food with long shelf life, food processors are using "super sanitation," which has efficiently eliminated the traditional microorganisms associated with spoilage before, but at the same time has enabled the minor flora to establish in the environment to contaminate the products. Finally, processors might be introducing equipment that are highly efficient in producing large volume of products, but may also be the microbiologist's nightmare for efficient cleaning and sanitation. As a result, the equipment harboring the microorganisms in dead spots remain unaffected by sanitation and serve as a source of inoculation to the products. Some of the examples used here may justify these assumptions.[5]

IV. INCIDENCE OF SPOILAGE OF VACUUM-PACKAGED REFRIGERATED FOOD

A. Spoilage of Unprocessed (Fresh) Beef by *Clostridium* spp.

Large-scale spoilage of vacuum-packaged refrigerated, unprocessed beef has been recognized recently.[5–9] The spoilage is characterized by accumulation of large quantities of H_2S-smelling gas and purge (liquid) in the bag within 2 weeks at 4°C, and loss of texture (soft) and color (cherry red) of meat, which in 10 to 12 weeks changes to greenish (Figure 20.1A). Enumeration by plating revealed ca. 10^8/ml leuconostocs colony-forming units (CFUs) in the purge. However, examination of a little purge under a phase-contrast microscope (see cover page photograph) revealed a large number of medium to large, thick, motile rods, some cells with large terminal spores (drumstick-shaped), as well as some leuconostoc-like cells (lenticular, small chains). The rods and the lenticular cells were Gram-positive. The rod-shaped bacterium was suspected to be a *Clostridium* species, but could not be cultured in agar and broth media recommended for *Clostridium* spp. Later, it was purified by heating the purge (from the package of a 12-week-old spoiled meat containing spores) at 80°C for 10 min and culturing the material at 15°C in a broth under anaerobic condition. Biochemical studies revealed it to be a new species and it was designated *Clo. laramie* NK1 (ATCC 51254). The cells are extremely sensitive to oxygen and grow optimally under a good anaerobic environment at 12 to 15°C, with a growth temperature range of –2 to 22°C. The species can sporulate and germinate at 2°C.

Inoculation of raw beef with these spores followed by vacuum packaging and refrigeration storage caused the samples to develop similar characteristics and the purge had the *Clostridium* cells. A limited in-plant sampling was done from the slaughtering area to the conveyor systems carrying the primal cuts for vacuum packaging (in a plant having the specific problem). Three of the 25 samples were positive for *Clo. laramie*, and all three were from the conveyor system at the vacuum-packaging area. The conveyor system used by this plant consisted of small links. In general, the microbiological quality of the meat was very good ($\approx 10^3$/g aerobic plate counts, APCs). It was suspected that the sanitation specifically designed to keep the microbial load low was good, but was unable to kill the spores, especially in the inaccessible joints (dead spot) and similar places in the links of the conveyor system. Thus, the fabricated meats were constantly inoculated with the spores (and cells), which, in the absence of competition, because of low associated bacteria, germinated or grew (or both) to cause large-scale spoilage (along with psychrotrophic *Leuconostoc* spp.). Subsequent studies with spoiled meat samples from several beef processors having similar problem in the U.S. have revealed that this incidence is not isolated and more than one strain of *Clo. laramie* is involved in spoilage.[5–8]

A similar spoilage of vacuum-packaged refrigerated beef in Europe was also reported to be caused by a similar *Clostridium* sp. and named *Clo. estertheticum*. This species also could not be enumerated by agar plating methods and was first detected microscopically.[7] In recent years, spoilage of vacuum-packaged refrigerated raw beef by *Clostridium* spp. has been reported from Canada and New Zealand.

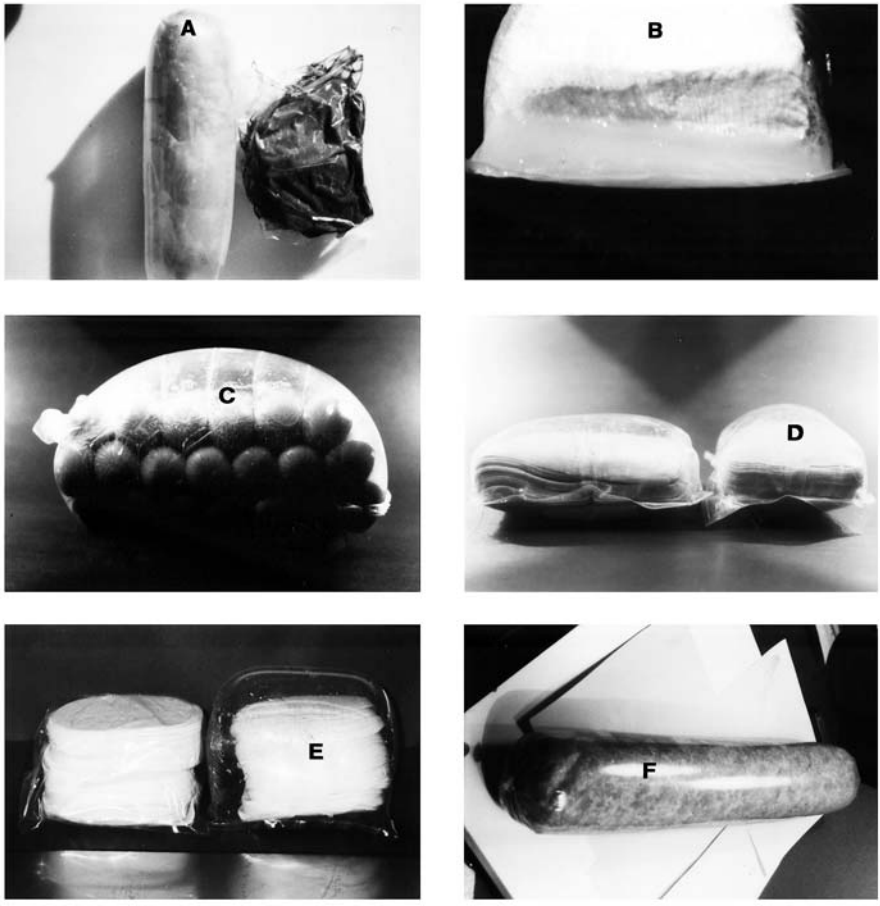


Figure 20.1 Bacterial spoilage of vacuum-packaged refrigerated foods. (A) Spoilage of beef by *Clo. laramie*: left, a spoiled sample showing gas accumulation; right, an unspoiled sample. (B) Spoiled tofu by a *Clostridium* sp. showing accumulation of liquid and gas. (C) Spoiled frankfurters and (D) spoiled luncheon meat predominantly by *Leuconostoc* spp. showing gas accumulation. (E) Spoiled sliced turkey roll by heterofermentative lactobacilli, leuconostocs, and *Ser. Liquifaciens*: right, gas accumulation (and pink discoloration); left, an unspoiled sample. (F) Spoilage of ground beef chub by heterofermentative lactobacilli showing gas accumulation.

B. Spoilage of Roasted Beef by *Clostridium* ssp.

Clostridium spp. have been isolated from at least two separate incidents of large-scale spoilage of vacuum-packaged refrigerated roasted beef produced commercially. The meats (10 to 12 lbs [4.5 to 5.5 kg] each) were roasted ≤ 160°F (71°C) (internal temperature) and then vacuum-packaged and refrigerated.[6,8] When the samples were received in the laboratory, they had large quantities of gas and purge but, because of the spices used in the meat, only a strong spicy smell was evident. Phase-contrast microscopy of the purge revealed large numbers of motile *Clostridium* cells, some

with typical terminal spores. Colonies of this bacterium were not obtained by isolation in specific agar media, but large numbers of leuconostoc colonies ($\cong 10^8$/ml of purge) were obtained. The spore-containing purge was heated and cultured to purify the *Clostridium* spp. Inoculation of the pure culture to rare roasted beef, followed by vacuum packaging and refrigeration, showed the formation of H_2S gas, change of meat color from light brown to pink, and accumulation of pink to red purge within 2 to 3 weeks. Examination of the purge under a phase-contrast microscope revealed the presence of *Clostridium* cells and spores. Some of these isolates seem to differ in some biochemical and physiological patterns when compared with *Clo. laramie* isolated from the spoiled raw beef (see Section IV-A).[5,7]

C. Spoilage of Pork Chops by *Clostridium algidicarnis*

This new species is associated with spoilage of cooked, vacuum-packaged pork meat products during storage at 4°C.[9] It produces an offensive spoilage odor.

D. Spoilage of Tofu by *Clostridium* spp.

A vacuum-packaged soybean milk curd product (Tofu) during refrigerated storage revealed gas and liquid accumulation in the bag and off-flavor (Figure 20.1B).[8] Microscopic examination of the liquid showed the presence of large numbers of motile rods, many with terminal oval spores. The strain was isolated in pure form and found to have some biochemical and physiological characteristics different from those of *Clo. laramie* NK 1 because it could be grown in agar medium under anaerobic condition. However, it is psychrophilic and does not grow at 25°C or above.

E. Spoilage of Unripened Soft Cheese by *Leuconostoc* spp.

Spanish-style soft cheese, made by coagulating pasteurized milk with lactic acid and rennet and collecting the curd and vacuum packaging, showed gas formation and liquid accumulation in the package within 30 d at refrigerated temperature. (The expected shelf life is 50+ d.)[8] The curd texture was soft and gassy. Examination of the liquid under a phase-contrast microscope revealed short chains of leuconostoc-like cells. Enumeration in pH 5.0 plating media revealed 10^{8-9} CFUs/g product. Purified cells from the colonies were Gram-positive, heterofermentative, and biochemically identified as *Leuconostoc* spp. The bacteria possibly get in the product during extensive handling of the product following pasteurization of milk. Even when they are initially present at a low level, during long storage they can multiply, form gas, and produce the defect. Any temperature abuse accelerates the spoilage.

F. Spoilage of Low-Heat-Processed Meat Products by *Leuconostoc* spp.

Many types of commercially processed, vacuum-packaged refrigerated meat products, following spoilage, were collected from local supermarkets. The products were

restructured ham steak, chopped and formed ham, chopped sliced ham, chunked and formed turkey ham, cooked luncheon meat (sliced), several brands of frankfurters (beef, chicken, and turkey), wieners, cooked and smoked Polish sausage, summer sausage, and hot-link sausage. The common symptoms were accumulation of large quantities of gas and cloudy purge (Figure 20.1C,D).[5–8] The products did not have any disagreeable odor. The pH ranged from 5.0 to 6.0. Some were low-fat products. Phase-contrast microscopy of the purge revealed predominantly the presence of leuconostoc-like cells. Colony enumeration of the purge revealed lactic acid bacteria counts of ca. 10^{8-9}/ ml, but lower APCs. *Bro. thermosphacta*, when present, was $<10^3$/ml. Biochemical tests revealed that the predominant lactic acid bacteria were *Leu. carnosum* and *Leu. mesenteroides*, and many could produce bacteriocins. It was suspected that bacteriocin-producing leuconostocs, by inhibiting growth of other Gram-positive bacteriocin-sensitive contaminants, grew preferentially and produced CO_2 and acid and were responsible for gross distension (or blowing) of the packs and release of liquid (purge). Most likely, the leuconostocs contaminated the products as postheat contaminants from the equipment and other sources during handling before vacuum packaging.[10]

G. Ammonia Odor in Turkey Roll

Low-fat (2%), high-pH (6.5) vacuum-packaged turkey breast rolls showed gas and liquid accumulation with a strong ammonia odor (diaper smell) when opened.[8] Examination of the liquid under a phase-contrast microscope showed predominantly leuconostocs-like cells, as well as motile single or small-chain rods and some lactobacilli-like cells. Purification and Gram staining revealed the leuconostocs-like and lactobacilli-like cells to be Gram-positive and the motile rods to be Gram-negative. APCs and lactic acid bacteria counts of liquid were ca. 10^8/ml. Biochemical analysis revealed the Gram-negative species to be *Ser. liquifaciens*, and Gram-positive bacteria were *Leu. mesenteroides* and *Lab. sake*. It was suspected that these predominant bacterial types contaminated the products after heat treatment and before vacuum packaging. *Leuconostoc* sp. produced gas (CO_2) and acid, and *Lab. sake* also produced acid but not gas because it is homofermentative. But the pH did not drop, owing to the high phosphate content used in this low-fat product. Subsequently, *Serratia* sp. grew and caused deamination of amino acids, thus releasing ammonia. The products also had a slight pink color, which could be due to partial reduction of metmyoglobin (see Section IV-J and Section IV-K).

H. Yellow Discoloration of Luncheon Meat

Vacuum-packaged cooked luncheon meat, prepared from chopped ham, developed yellow-colored spots within 3 to 4 weeks of storage at 40°F (4.4°C).[11] Microbiological analysis indicated the presence of high numbers of *Enterococcus faecium* ssp. *casseliflavus*, which was able to survive at 71.1°C for 20 min. Thus, the species probably survived cooking. The yellow discoloration was suspected to be due to the production of a carotenoid substance by the bacterial species.

I. Gray Discoloration of Turkey Luncheon Meat

Turkey luncheon meat slices prepared mainly from dark meat developed gray spots or patches within 2 to 3 d during aerobic storage at refrigeration temperature.[8] The causative bacteria was isolated and identified to be an H_2O_2-producing strain of *Lactobacillus* species. The product formulation contained ca. 1% lactate. It is suspected that the bacterial strain under aerobic conditions of growth utilized lactate to produce H_2O_2 (lactate + O_2 → pyruvate + H_2O_2, reaction catalyzed by L-lactate oxidase). H_2O_2 then oxidized the myoglobin to produce a white-gray color. The H_2O_2-producing strain could come as postheat contaminants or survive low-heat treatment, as some lactobacilli are thermoduric. Under vacuum storage, the strain does not produce H_2O_2 to cause discoloration.

J. Pink Discoloration of Sliced, Chopped, and Formed Roast Beef

Vacuum-packaged chopped and formed low-fat roast beef slices during refrigeration storage for 1 to 2 weeks developed pink to red patches over the normal brown color.[8] Initially, the fresh product had a pH of 5.8 to 6.0 and a slight off-odor. Within 4 to 5 weeks at refrigerated temperature, the slices developed a strong fishy to putrid odor; no H_2S was detected, and the pH remained ca. 6.0. The meat color changed to deep pink to red with a dark-red colored purge, but there was a very small amount of gas accumulation. Microbiological enumeration revealed ca. 9×10^9/ml purge of APC and 1×10^9/ml purge of lactic acid bacteria (leuconostocs or lactobacilli). Examination of the purge under a phase-contrast microscope revealed leuconostocs- and lactobacilli-like cells, as well as motile rods. Streaking on xylose-lysine iron agar plates helped isolate three types of colonies of Gram-negative rods, which were biochemically identified as *Hafnia alvei, Proteus vulgaris*, and *Ser. liquifaciens*. Inoculation of the three isolates in roast beef, followed by vacuum packaging and storage at refrigerated temperature, revealed that all three Gram-negative isolates could change the brown color of roasts to pink or red (like rare roast beef) with a red-colored purge, but *Pro. vulgaris* changed the meat color to cherry red. The packages inoculated with *Ser. liquifaciens* also had slight gas. All three Gram-negative isolates produced small amounts of H_2S. Analysis of absorption spectra of the extract revealed that the color change occurs because of the partial reduction of metmyoglobin.

The lactic acid bacteria as well as the Gram-negative species isolated from these samples were heat-sensitive psychotrophic and facultative anaerobic. They definitely entered the product as postheat contaminants, indicating poor sanitation in the production facilities. The absence of large amounts of gas in this product, as opposed to some products described before, could be due to the presence of very little metabolizable carbohydrate. The reason for the pH to remain unchanged (≅ pH 6.0) during deamination of amino acids could be the use of high amounts of phosphate in the formulation to bind water.

K. Gas Distension and Pink Discoloration of Sliced Turkey Rolls

Sliced low-fat turkey rolls (initial pH 6.5) were packaged by modified atmosphere (drawing vacuum and then flushing a mixture of 30% CO_2 and 70% N_2 in small

amounts) so that the packages remained slightly loose. The product is expected to have an 8-week shelf life at refrigerated temperature. By 5 weeks, many packages developed gross distension with gas, little to moderate amounts of purge, and pinkish-red discoloration from the normal gray-white color of breast meat (Figure 20.1E).[8] The intensity of the color was more inside the slices than at the rim. The gas had an off-odor (like wet socks), but no H_2S was detected. Soon after opening a bag, the color of meat slices changed to normal. Vacuum packaging the meat slices again changed the color in 8 to 12 h to pinkish red. Addition of ascorbic acid to the normal colored meat slices gave the similar pinkish-red color quickly (1 to 2 h). Examination of the liquid under a phase-contrast microscope revealed leuconostocs-like and lactobacilli-like cells, as well as motile rods. Colony enumeration revealed the presence of lactic acid bacteria ca. 10^{7-8}/g and *Enterobacteriaceae* ca. 10^6/g. While lactic acid bacteria were predominantly *Leu. carnosum* (heterofermentative) and *Lab. curvatus* (homofermentative), *Enterobacteriaceae* were *Ser. liquifaciens*. Inoculation studies revealed that *Ser. liquifaciens* was important for the off-flavor. The pink color was due to formation of reduced metmyoglobin; however, it is not clear how it formed. It seems that psychrotrophic Gram-positive and Gram-negative bacteria, entering the product as postheat contaminants, grew during storage and reduced the O-R potential of the products. This changed the metmyoglobin from reversible oxidized to reduced state, and color from gray-white to pink. In air (oxygen), metmyoglobin reversed from reduced to oxidized state, changing the color from pink to gray-white. Absorption spectrum studies with a spectrophotometer of extracted materials also supported the conclusion that the pink color was due to a reduced form of metmyoglobin. A large amount of gas was produced by *Leu. carnosum* from the metabolism of glucose used in the formulation. Because the product had fairly large amounts of phosphate, pH did not change, even from the growth of lactic acid bacteria; thus, there was only a small amount of purge present in the bags.

Examination of the production facility revealed that the cooked products were contaminated by these bacteria mainly from the slicer and the conveyors between the slicer and packaging machine. However, the initial contamination level was very low (<10/g). High numbers were reached during storage. It was also revealed by the processor that during distribution from the warehouse to stores in a van, the product temperature could be as high as 50 to 55°F (10 to 12°C) for 6 to 8 h; this could also result in rapid growth of psychrotrophs, causing reduction in shelf life.

L. Gas Distension (Blowing) of Ground Beef Chubs

Vacuum-packaged low-fat ground beef chubs were found to accumulate large quantities of gas within a few days of refrigerated storage (Figure 20.1F). Initially (within 1 week of storage), there was a slight off-odor (no H_2S) in the gas or meat, the meat pH was 5.6, and microbiological analysis revealed large numbers (1 to 4×10^7/g) of psychrotrophic lactic acid bacteria, mainly *Leu. mesenteroides* and two atypical *Lactobacillus* spp. (as determined from biochemical and physiological reaction patterns). APCs were $\leq 1 \times 10^5$/g, and Gram-negatives were $\leq 10^3$/g. The most predominant *Lactobacillus* sp. is heterofermentative, has atypical morphology, and its

biochemical profile (by API system) does not match that of any known species. Following storage of the meat for ca. 6 weeks at refrigerated temperature, psychrotrophic lactic acid bacterial counts increased to ca. 1.5×10^8/g (with APC $\cong 2.7 \times 10^7$/g), the pH increased to 6.2, the gas had slight H_2S, and the meat had a strong putrid odor. It was suspected that initial gas formation was due to metabolism of carbohydrates in meat by heterofermentative psychrotrophic lactic acid bacteria. During extended storage, lactic acid bacteria as well as other bacteria (especially Gram-negative) assimilated amino acids, including sulfur-containing amino acids, and produced the offensive odor.

M. Egg Odor in Refrigerated Fresh Chicken Meat Products

Skinless and boneless chicken breast meat were diced and marinated (the mixture contained different ingredients, including 1.2% salt and 1.8% sodium lactate), vacuum packaged, and stored at 30°F (–1.1°C). The pH of the product was 6.0 to 6.2. Within 7 d, the products developed a mild egg odor that increased in intensity during the expected storage life of 28 d.

Vacuum-packaged samples (4 d and 24 d old) were received in ice pack containers and analyzed within 24 h. Duplicate samples were tested for each microbial group examined. There was no gas or exudate accumulation in the bags, but in the 24-d-old samples the bags were slightly loose. Following opening, the fresh samples had a chicken odor, but the old samples had a distinct egg odor and were positive for the presence of H_2S (tested with lead acetate). The pH of both groups of product was 6.0. The bacterial groups enumerated were APCs (35°C for 2 d) and psychrotrophic counts (10°C for 7 d), both with plate count agar; lactic acid bacteria (35°C for 2 d) in MRS-agar (adjusted to pH 5.0); coliforms (35°C for 1 d) in violet red bile agar; and *Enterobacteriaceae* (35°C for 2 d) in violet red bile glucose agar media. Aliquots from appropriate dilutions were pour-plated in duplicate for each dilution.

The results (Table 20.1) revealed that in the 4-d-old products, the levels of five bacterial groups were not unexpectedly high for a diced meat product. Similar levels for APC were also obtained in 1-d-old products, which were tested in the processor's laboratory. After 24 d at 30°F (–1.1°C), both APCs and psychrotroph counts increased but remained below the usually accepted spoilage detection level (ca. ≥

Table 20.1 Change in CFUs/g of Several Bacterial Groups in a Refrigerated Chicken Meat Product during Storage

Bacterial Groups	CFUs/g[a]	
	Fresh (4 d)[b]	Old (24 d)
Aerobic plate count	5.9×10^4	5.8×10^5
Lactic acid bacteria	4.9×10^4	3.5×10^4
Psychrotrophic bacteria	7.0×10^4	4.5×10^5
Coliforms	4.0×10^3	7.0×10^2
Enterobacteriaceae	5.6×10^3	1.5×10^2

[a]Average of two samples.
[b]Fresh samples, but not the old samples, had ca. 6×10^2 *Proteus*.

10^7/g). However, levels of coliforms and *Enterobacteriaceae* decreased during storage. Interestingly, fresh samples had ca. 6×10^2/g *Proteus*, but 24-d-old samples did not have any in a 10^{-1} dilution. It was suspected that cells of *Enterobacteriaceae*, including *Proteus* sp., were killed during storage by the combined action of 1.8% lactate, 1.2% salt, and 30°F (–1.1°C). Their proteolytic enzymes (exocellular and endocellular) were able to break down proteins and sulfur-containing amino acids to produce a small amount of H_2S associated with egg odor. To overcome such problems, an important objective will be to keep the level of *Enterobacteriaceae*, as well as the level of Gram-negative bacteria, very low.

N. Off-Odor in Frozen Chicken Meat Product

Skinless and boneless chicken thigh meat was diced and marinated (the mixture contained different ingredients, including 3% sodium lactate and 1.8% salt). The products (pH 6.3) were vacuum packaged and frozen to –20°C. The product, expected to have a shelf life of 90 d or more, is thawed by customers immediately before use. When thawed during storage, the products gave an off-odor, which increased in intensity with storage period.

Vacuum-packaged meat samples consisting of frozen unmarinated and marinated and frozen stored for 25 d and 100 d were enumerated for the levels of several bacterial groups by the methods described in Section IV-M). The packages of the marinated products were loose and the thawed meat had an off-flavor, which was distinct in the 100-d stored products. The results (Table 20.2) revealed that unmarinated product had low levels of four bacterial groups. In contrast, the 25-d stored products had high levels of all four groups. In the 100-d storage products, the counts of all four bacterial groups dropped by two to three log cycles.

It was suspected that the unmarinated product was heavily contaminated during the process involving marination. Many of these were Gram-negatives (psychrotrophs, coliforms, *Enterobacteriaceae*). During frozen storage, in the presence of high lactate and salt, many of them were killed. Some of their enzymes were able to catalyze reactions slowly at –20°C and caused slight proteolysis (and maybe lipolysis), producing flavor compounds that could be identified after the meat was thawed (see Chapter 21). To overcome the problem, it will be important to adopt good sanitary practices and proper temperature control during processing and before freezing the product.

Table 20.2 Change in CFUs/g of Several Bacterial Groups in Frozen Chicken Meat Product during Storage

Meat Types	Days at –20°C	CFUs/g[a]			
		APC	Psychrotrophs	Coliforms	Enterobacteriaceae
Unprocessed	7	1×10^4	3×10^4	5×10^1	2.8×10^3
Processed	25	1×10^7	1.9×10^7	1.1×10^4	1×10^5
Processed	100	4×10^4	3.2×10^4	1.4×10^2	1.1×10^2

[a]Average of two or more samples.

O. Gas and Slime Development in Vacuum-Packaged Smoked Salmon Products

Processors have reported different types of spoilage in vacuum-packaged refrigerated smoked salmon hot dog and deli-type products. The hot dog packages had large amounts of gas and very thick, slimy, yellow-whitish purge. The deli-type packages had very little gas but large volumes of cloudy purge. Examination revealed that H_2S was absent in the gas, and pH of the purge was ca. 4.8 for both types of samples. Phase-contrast microscopy of the purge revealed the presence of coccoid to lenticular cells in small chains in the hot dogs but different-sized rods in the deli samples. Microbiological enumeration of the purge revealed the presence of lactic acid bacterial CFUs more than 10^{10}/ml. Biochemical analysis revealed the predominant coccoid and lenticular isolates in hot dogs to be *Leu. mesenteroides* ssp. *dextranicum* and the predominant rod-shaped isolates in deli-style products to be *Lab. sake.* Both bacterial isolates were killed at 71°C in 5 min (the products were processed at 72°C, internal temperature) but are able to multiply at 4°C.

It was suggested that both lactic acid bacterial strains were present in the packaged products as postheat treatment contaminants. The ingredients had both glucose and brown sugar in fairly high concentrations, which facilitate both psychrotrophic facultative anaerobic strains to multiply at refrigerated storage temperature. The heterofermentative *Leuconostoc* strain produced gas and dextran (from sugar). Both reduced the original product pH of 5.6 to 4.8, causing the loss of bound water to produce purge in the bags. The recommendations are to replace the sugar in the formulation, use proper sanitation to reduce postheat contamination load, maintain the storage temperature below 4°C, and reduce temperature abuse.

V. CONCLUSION

The examples in this chapter describe how products are contaminated with different types of spoilage bacteria, many as postheat contaminants. Complicated machineries, handling of large volumes of products, and a desire for low initial microbial load by good sanitation have probably selected out those bacteria that can establish in the facilities and contaminate the products. In the absence of competition and being psychrotrophic and anaerobic or facultative anaerobic, even from a low initial contamination level the bacteria can multiply and reach high levels during long storage to cause spoilage. In the absence of effective preservatives as well as because of temperature abuse during storage, transport, and display, they can grow more rapidly and cause rapid spoilage of the products. In some situations, poor sanitation (contamination with Gram-negatives) of the facilities was observed. Table 20.3 summarizes some general characteristics of predominant spoilage bacteria in vacuum-packaged refrigerated processed foods. It is also important to recognize that because processed products are stored at refrigerated or frozen temperature for a long time (60 to 100 d or more), some enzymes of the contaminating microorganisms can cause spoilage, even in the absence of live cells. This is discussed in Chapter 21.

Table 20.3 Spoilage Characteristics of Some Predominant Bacteria in Vacuum-Packaged Refrigerated Foods

Spoilage Characteristics[a]	Cell Morphology[b]	Most Likely Bacteria[c]
Large volume of gas and purge, no H_2S, not much off-odor, purge pH ≤ 5.5	Coccoid to lenticular, small chains, Gram +ve	*Leuconostoc* spp.
Little gas and purge, no H_2S, not much off- odor, purge pH ≤ 5.	Rods, some in chains, nonmotile, Gram +ve	Homofermentative *Lactobacillus* spp.
Large gas, little purge, H_2S +/–, off-odor, purge pH ≤ 6	Rods, some in chains, nonmotile, Gram +ve	Heterofermentative *Lactobacillus* spp.
Little gas and purge, NH_3 odor, purge pH ≥ 7.5	Small rods, motile, Gram –ve	*Serratia* spp.
Little gas and purge, putrid odor, purge pH ≥ 8.0	Small rods, motile, Gram –ve	*Hafnia*, other *Enterobacteriaceae*
Large volume of gas and purge, H_2S odor, purge pH ≥ 7.5	Thick rods of different sizes, motile, some with terminal spores, Gram +ve	Psychrotrophic *Clostridium* spp.

[a]There are some characteristics commonly found in the early stage of spoilage of a product. However, a sample can be spoiled by more than one type, especially in the early stages and can show mixed characteristics.

[b]Morphology is examined with a drop of purge under a phase-contrast microscope. With practice, an almost presumptive positive identification can be made in minutes.

[c]Except for spores of *Clostridium* spp., all are sensitive to low-heat treatments (pasteurization). Their presence in heated products results mostly from postheat contamination and on the product surface.

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QUESTIONS

1. Define psychrophilic and psychrotrophic microorganisms. Discuss why psychrotrophs are important in food spoilage.
2. Briefly discuss the reasons for the current popularity of refrigerated foods.
3. What are the possible causes of microbiological problems in refrigerated foods?
4. List six bacterial genera or species that are currently designated as new spoilage bacteria. Explain the possible reasons for their becoming important in the spoilage of refrigerated foods.
5. A processor recently started producing a new vacuum-packaged, low-heat processed, low-fat meat product with an expected shelf life of 60 d at 40°F (4.4°C). He found that ca. 10% of the product was getting spoiled within 30 to 40 d. The characteristics were accumulation of gas and purge (cloudy), with acidic and cheesy odor. He asks you to help him determine the causes and resolve the problem. Explain briefly, in a stepwise fashion, how you plan to proceed in order to understand the problem (causes, sources, sequence of events, etc.] and solve it.
6. Answer Question 3 to Question 6 in Chapter 3 with the current understanding from this chapter on the pathogen problems in the listed foods. Suggest their route of contamination of the respective products and the sequences that lead to the problem in each case.

CHAPTER 21

Food Spoilage by Microbial Enzymes

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I. INTRODUCTION

The metabolism of food nutrients, principally carbohydrates, nitrogenous compounds, and lipids, by spoilage bacteria enables the cells to increase in number as well as produce metabolites that can adversely reduce the acceptance quality of a food. A food is considered spoiled when the changes are detectable and the microbial population has reached to ca. 10^{7-9}/ml, /g, or /cm^2. The changes are brought about by the catalytic actions of a large number of microbial enzymes. Most microbial enzymes are intracellular and act on the nutrients that can be transported inside the cells through several transport mechanisms. A bacterial cell contains many enzymes, many of which are intracellular and some are extracellular. Intracellular enzymes are involved in the metabolism of small nutrient molecules of food that are transported inside the cells. Many intracellular enzymes can also act on intracellular large molecules, such as endonucleases, mucopeptidase, lipases, and proteinases. Extracellular enzymes, after synthesis, either remain bound to the cell surface or are

excreted in the food environment. Many of the latter group can hydrolyze large nutrient molecules of food (e.g., polysaccharides, proteins, and lipids) to small molecules before they are transported into the cells (see Chapter 7 and Chapter 11).

Most foods have some amounts of low-molecular-weight metabolizable carbohydrates (mono- and disaccharides and their derivatives, such as glucose-6-phosphate), nitrogenous compounds (small peptides, amino acids, nucleosides, nucleotides, urea, creatinine, trimethylamine oxide), free fatty acids, and some organic acids (lactic, citric, and malic acids). Many spoilage microorganisms, particularly spoilage bacteria, are able to utilize the low-molecular-weight food components to reach a population of 10^{7-9}/g, /ml, or /cm^2 food and cause detectable food spoilage. Thus, the supply of extra nutrients from the hydrolysis of the macromolecules of foods by bacterial extracellular enzymes is not necessary for the onset of spoilage of many foods. In fact, studies with *Pseudomonas* spp. and *Bacillus* spp. show that in the presence of low-molecular-weight nitrogenous compounds, the synthesis of extracellular proteinases is repressed. When the supply of the small molecules is used up, the mechanism is derepressed, leading to the synthesis and excretion of extracellular proteinases. The proteinases hydrolyze the large protein molecules of food to produce small peptides and amino acids for transport and endogenous metabolism in the cells, which in turn intensify the spoilage. In general, microbial food spoilage from the metabolism of low-molecular-weight nutrients occurs at the early stage of microbial growth; spoilage from the breakdown of macromolecules by extracellular enzymes appears late in the sequence of events.[1,2]

After microbial cells die normally or are killed by nonthermal treatments so that the intracellular and extracellular enzymes are not inactivated or destroyed, the enzymes can cause food spoilage even in the absence of viable cells or growth of microorganisms. Many microbial cells in a food that are subjected to freezing (then thawing), drying (then rehydrated), modified atmosphere or vacuum packaging, refrigeration, high hydrostatic pressure, electric pulse field, or high-intensity light, or are exposed to some preservatives, may die and undergo autolysis to release intracellular enzymes. If these foods are stored for a long time under conditions that favor catalysis of one or more intra- and extracellular enzymes, they can undergo spoilage. Ripening (not spoilage) of cheddar cheese (at low temperature and low A_w) is a good example wherein following death, bacterial intracellular enzymes are involved in the breakdown of milk nutrients, not to cause spoilage but to impart desirable product characteristics. In most foods, the initial microbial population is generally low and spoilage by microbial enzymes in the absence of growth may not be of practical significance. However, if a product is heavily contaminated with a high initial microbial load (in the absence of growth) and then subjected to a nonthermal treatment that kills microorganisms but does not inactivate enzymes, spoilage of the food by microbial enzymes can occur (Chapter 20). In thermally processed foods, several heat-stable enzymes of the microorganisms retain their activity even after the producer cells are killed. During subsequent storage of the food under favorable conditions, these enzymes can break down the food nutrients to cause spoilage. Among the heat-stable enzymes, some extracellular proteinases, lipases, and phospholipases of several psychrotrophic bacteria found in food cause spoilage of thermally processed dairy products.[1,2]

II. CHARACTERISTICS OF HEAT-STABLE ENZYMES OF PSYCHROTROPHIC BACTERIA

Raw milk, between production and pasteurization in commercial operations, is usually stored for 1 to 2 weeks at refrigerated temperature (<7°C). Psychrotrophic bacteria in the raw milk, coming from water, equipment, and environment, can multiply during storage. Depending on initial numbers, bacterial species and strains, temperature and time of storage, and extent of temperature abuse, the population can reach the level of producing sufficient amounts of extracellular heat-stable enzymes. Even though raw milk may not be spoiled, the heat-stable enzymes produced can cause spoilage of heat-treated dairy products manufactured from this raw milk. Heating, such as pasteurization and ultrahigh temperature treatments (UHT), kills psychrotrophic bacteria but does not inactivate heat-stable enzymes.

In raw milk, heat-stable proteinases, lipases, and, to some extent, phospholipases produced by several psychrotrophic Gram-negative bacteria are considered to be of major economic importance because of the spoilage potential of the products. Species from genera *Pseudomonas (Pseudomonas fluorescens, Pse. fragi), Aeromonas, Flavobacterium, Shewanella (Alteromonas), Serratia,* and *Acinetobacter* produce heat-stable extracellular proteinases, and *Pseudomonas, Alcaligenes, Shewanella, Acinetobacter*, and *Serratia* produce heat-stable lipases. *Pseudomonas* spp. also produce heat-stable phospholipases. Many species from these genera are normally present in raw milk, meat, fish, and other food products. During refrigerated storage, psychrotrophs are able to grow and produce heat-stable enzymes in foods.[1–4]

Production of heat-stable proteinases and lipases in milk by some of these bacteria, especially *Pseudomonas* spp., and the characteristics of these enzymes have been well studied. The results show that different species and strains produce proteinases and lipases that differ in molecular weight and activity. Thus, a highly active proteinase or lipase produced by a *Pseudomonas* strain can produce extensive proteolysis or lipolysis, even at 10^{5-6} cells/ml milk. But another strain may need to reach to 10^9 cells/ml to produce similar changes. The enzymes can be produced in raw milk at refrigerated temperature (1 to 7°C) in sufficient quantities to hydrolyze proteins and lipids in detectable levels within 3 to 7 d. The catalytic activity of the proteinases is highest between pH 6.0 and 7.0, with a pH range between 5.0 and 9.0. Pasteurization of milk (at 63°C for 30 min or 71°C for 15 sec) results in a loss of 6 to 36% activity and, even after heating at 121°C for 10 min, some activity of the proteinases is retained. UHT treatment (140 to 150°C for 1 to 5 sec) fails to completely inactivate proteinases produced by some species and strains of *Pseudomonas* and other psychrotrophs. UHT-treated milk can thus be spoiled by the residual activity of the proteinases during storage. Lipases are only partially inactivated by pasteurization or by heating (in cream) at 90°C for 2 min. They are generally inactivated by UHT treatment. Lipases from some *Pseudomonas* strains retain sufficient activity even after heating at 100°C for 10 min.

Heat-stable proteinases of psychrotrophic bacteria differ in their substrate specificity and rate of substrate degradation. Proteinases of *Pseudomonas* spp. preferentially degrade casein of milk by a different mechanism. Proteinases from some species or strains initially degrade β-casein, whereas proteinases from other species

or strains initially degrade κ-casein. With time, they can also degrade other casein fractions. Proteinases from psychrotrophic species or strains of *Flavobacterium, Aeromonas,* and *Serratia* also showed initial differences in the degradation of β- and κ-caseins. α-Casein is degraded last by all strains.[1–4]

Lipases of psychrotrophic bacteria differ in their specificity toward lipids. A lipase from a strain of *Pse. fragi* specifically hydrolyzed fatty acids from positions 1 and 3 of the triglycerides, whereas another lipase from a second strain hydrolyzed only at position 1. Similar specificities have been observed with extracellular lipases from *Pse. fluorescens*. Some lipases of psychrotrophic bacteria equally hydrolyze all three fatty acids.

Among the phospholipases produced by psychrotrophic Gram-negative bacteria, phospholipase C from *Pseudomonas* spp. has relatively high heat stability. It is not destroyed by pasteurization. Phospholipase C from *Pse. fluorescens* is more active against phosphatidylethanolamine than other phospholipids.[1–4]

III. SPOILAGE OF FOODS WITH HEAT-STABLE MICROBIAL ENZYMES

The presence of heat-stable extracellular enzymes of psychrotrophic bacteria in raw milk can cause spoilage of dairy products made from it. In addition, when these dairy products are used as ingredients to make other food products, the action of heat-stable enzymes can also reduce their acceptance qualities. Several examples are used here to emphasize the spoilage potential of these enzymes.[1,2,5–7]

A. Pasteurized Milk

Heat-stable proteinases and lipases of psychrotrophic bacteria are not inactivated by pasteurization and can cause proteolysis of casein and lipolysis of milk lipids to produce flavor defects. However, under normal short-term refrigerated storage, these defects may not be enough to detect. In addition, psychrotrophic bacteria contaminating milk after pasteurization can also multiply during storage and cause spoilage, especially when the milk is either stored for a long time or temperature abused. In that event, it is difficult to differentiate the role of bacterial growth and heat-stable enzymes in spoilage.

B. Ultrahigh Temperature (UHT)-Treated Milk Products

UHT-treated milks, heated at 140 to 150°C for 1 to 5 s, are considered commercially sterile products with a shelf life of 3 months at 20°C. Spoilage of these products during storage at 20°C has been observed in the form of bitter flavor, sediments, and gel formation, due to the action of heat-stable proteinases and rancid flavor from the action of heat-stable lipases. Generally, the changes produced by proteinases are more predominant than those associated with lipases. However, in the presence of heat-stable phospholipases, the lipolysis caused by lipases can be detected. It is speculated that phospholipases degrade the phospholipids in the membrane of fat globules and increase the susceptibility of fat to lipases.

The time of spoilage of UHT-treated milk by heat-stable proteinases is dependent on the numbers and strains of *Pseudomonas* species growing in the raw milk. In a controlled study, UHT-treated milk was prepared from raw milk inoculated with a *Pse. fluorescens* strain and grown to 8×10^5 to 5×10^7 cells/ml. During storage at 20°C, the product with 5×10^7 cells/ml gelled in 10 to 14 d; the product with 8×10^6 cells/ml gelled in 8 to 10 weeks; and the product with 8×10^5 cells/ml did not gel in 20 weeks but had sediments. Raw milk whose caseins have been highly degraded by proteolytic enzymes may even be unstable to high heat treatment and thus cannot be used for the manufacture of UHT-treated milk.

C. Cheeses

Proteolytic activity by the extracellular proteinases of psychrotrophic bacteria in raw milk was reported to reduce cheese yield and increase the levels of nitrogenous compounds in whey. Depending on the proteolysis of caseins, the loss in cheese yield can be as high as 5%. The loss was directly related to the storage time of the raw milk and psychrotrophic counts. In addition, the heat-stable proteolytic enzymes were associated with increased proteolysis of cheeses (especially soft cheeses such as cottage cheese), lower flavor quality, and higher texture problems in cheddar cheese. Lipases have also been implicated in the development of off-flavor in cheese.

D. Cultured Dairy Products

Buttermilk and yogurt, made from milk that has had substantial growth of psychrotrophic bacteria before heat treatment, generally have poor texture and rapidly develop off-flavor during storage, even though they have low pH and are stored at ca. 10°C. These defects are attributed to bacterial heat-stable proteinases.

E. Cream and Butter

Cream and butter are more susceptible to spoilage by heat-stable lipases than by proteinases. Extracellular lipases of psychrotrophic bacteria preferentially partition with the cream phase of milk, increasing their concentrations in cream. Lipases are responsible for off-flavor development in cream. A cream that has undergone lipolysis foams excessively and takes a longer time to churn during processing of butter. The butter prepared from such cream is susceptible to rancidity more quickly.

Butter containing residual heat-stable bacterial lipases undergoes rapid lipid hydrolysis even during storage at –10°C. Lipases that preferentially release short-chain fatty acids (C4 to C8), with and without long-chain fatty acids, cause the most off-flavor in butter.

F. Milk Powder

The activity of heat-stable bacterial proteinases and lipases present in raw and pasteurized milk is not denatured during the manufacture of spray-dried milk powder. The low A_w prevents these enzymes from degrading the proteins and lipids in dry

milk. Powdered whole milk, nonfat milk, and whey are used as ingredients in a wide variety of foods that can have high amounts of proteins and lipids and are expected to have a long shelf life. Bakery products, ice cream, desserts, processed meat products, chocolate, cheese products, and condensed dairy products are some of the foods in which dry milks are used. These products can develop off-flavor and texture defects from the action of heat-stable proteinases and lipases during storage.

IV. SPOILAGE OF FOODS BY MICROBIAL ENZYMES AT LOW TEMPERATURE

Limited studies have shown that bacterial extracellular proteinases are not directly associated with development of off-flavor in fish and meat. The initial off-flavors associated with degradation of nitrogenous compounds develop from the bacterial metabolism of nonprotein nitrogenous (NPN) compounds present in these products. However, the bacterial extracellular proteinases once produced are able to act on tissue proteins and cause texture defects (such as slime formation). This probably occurs when the NPN compounds have been used up and the population has reached to ca. 10^8/g or /cm^2 of the products. Protein hydrolysis by bacterial proteinases favors the development of a higher degree of putrefactive changes. Some proteinases of *Pseudomonas fragi* reduce oxymyoglobin and discolor meat. The enzymes probably hydrolyze the globin part or the peptide chain of the myoglobin, which alters the reactivity of the heme group, leading to discoloration of meat. Muscle lipids can also be hydrolyzed by bacterial lipases, causing flavor defects in meat and fish. However, because lipids are susceptible to rancidity caused by lipases of the flesh and by autooxidation, the contribution of bacterial lipases is probably very little.

The heat stability of the bacterial proteinases and lipases associated with flavor and texture defects in raw meat and fish is not known. However, these enzymes are produced by the same psychrotrophic bacteria, namely, *Pseudomonas, Aeromonas*, and similar species, that are known to produce extracellular heat-stable enzymes. It can be assumed that some of these enzymes are heat stable. It will be important to determine whether these enzymes are potentially able to cause spoilage of low-heat-processed (pasteurized) meat products, some of which are expected to have a shelf life of 100 d.

In recent years, some specialty raw chicken meat products, that were stored at refrigerated temperature (–1 to 0°C for 28 d) or at frozen temperature (–20°C up to 90 d), were reported to develop distinct flavor defects before the expiration dates. Microbiological analysis of the freshly prepared products and the products with flavor defects during storage led to the suspicion that bacterial enzymes active at low temperatures might be associated with the flavor defects. The enzymes could be the exoenzymes, produced by the bacteria in a product while it was still fresh, which acted on specific food components during long storage and produced the flavor components in detectable amounts. At low temperatures, bacterial cells can also die, lyse, and release intracellular enzymes. Some of these enzymes can be active at low temperature to produce a defect that can be detected during long storage. It is likely that in the future, commercial production of many such value-added

specialty products with a long shelf life will increase. It will be important to recognize the problem and determine the cause to overcome such product defects. These aspects are discussed with actual incidents in Chapter 20.

V. CONCLUSION

Enzymes of microorganisms either secreted outside or released from inside following lysis of the cells can cause spoilage of specific foods, even in the absence of live cells or cell growth. Some of these enzymes can be resistant to physical and chemical treatments given to food and can be active at the storage conditions of the food. As our likings for the refrigerated foods increase and the foods are stored for a longer time, problems associated with these enzymes will increase. There is a need to determine the presence of live cells as well as their enzymes to predict the shelf life of foods. In Chapter 22, some of the methods currently used to determine shelf life as well as some new approaches are discussed.

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QUESTIONS

1. Discuss the role of intracellular enzymes of spoilage bacteria in food spoilage.
2. Describe the role of extracellular enzymes of spoilage bacteria in food spoilage.
3. Briefly discuss the production and characteristics of heat-stable enzymes by psychrotrophic Gram-negative bacteria. List three bacterial genera that produce heat-stable proteinases and lipases.
4. Briefly describe the problems associated with heat-stable microbial enzymes in UHT milk, cottage cheese, cheddar cheese, and cream.

5. Explain possible spoilage problems from using nonfat dry milk and cream made from a batch of raw milk in which a *Pse. flourescens* grew to 5×10^6 cells/ml; nonfat dry milk is used to make yogurt and cream to make sour cream.
6. What are the possibilities for heat-stable enzymes of spoilage bacteria to cause spoilage of a low-heat-processed, vacuum-packaged refrigerated meat product with a shelf life of 15 weeks?
7. Nowadays, many foods are stored under refrigerated or frozen temperature (≤–20°C). Discuss possible spoilage problems in these foods because of microbial enzymes in the absence of microbial growth.

CHAPTER 22

Indicators of Microbial Food Spoilage

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I. INTRODUCTION

Microorganisms cause food spoilage in two ways. The first, the more important one, is through the growth and active metabolism of food components by the live cells. The other is produced, even in the absence of live cells, by their extracellular and intracellular enzymes that react with the food components and change their functional properties, leading to spoilage. The loss of food by microbial spoilage has economic consequences for producers, processors, and consumers. With the increase in world population, loss of food by microbial (and nonmicrobial) spoilage means that less food is available for the hungry mouth. To fight world hunger, efforts should be directed not only to increase food production but also to minimize spoilage so that enough food is available for consumption. Many preservation methods have been devised to reduce microbial spoilage and discussed in Section VI. Under certain methods of preservation, both raw and partially processed (semipreserved, perishable, nonsterile) foods are susceptible to microbial spoilage. This is more evident in foods that are expected to have a long shelf life. To reduce loss of raw and partially

processed foods by microbial spoilage, two points are important. One is to predict how long a food, following production, will stay acceptable under the conditions of storage normally used for that food, i.e., what will be the expected shelf life under normal conditions of handling and storage. The other is to determine the current status, with respect to spoilage, of a food that has been stored for some time. This information needs to be available well before a food develops obvious detectable spoilage and therefore becomes unacceptable.

Many criteria have been evaluated for their efficiency as indicators to predict expected shelf life, as well as to estimate stages of microbial food spoilage. These criteria or indicators can be grouped as sensory, microbiological, and chemical (specific microbial metabolites). Sensory tests (e.g., changes in color, odor, flavor, texture, and general appearance), although easy and quick to perform, have several drawbacks as indicators, especially if used alone. Changes in texture and flavor generally appear at the advanced stages of spoilage. Odor changes can be masked by the spices used in many products. Odor changes from volatile metabolites may not be detected in a product that is exposed to air, as compared with the same product in an airtight package. Color changes, such as in meat exposed to air, may not be associated with microbial growth. Finally, individuals differ greatly in their perception of organoleptic criteria. However, sensory criteria can be used advantageously along with microbiological or chemical criteria, or both.

Studies by many researchers have clearly revealed that a single microbiological or chemical test is not effective in predicting either the shelf life of a product or its spoilage status. The contributing factors in microbial spoilage of a food include the type of product, its composition, methods used during processing, contamination during processing, nature of packaging, temperature and time of storage, and possible temperature abuse. Because these factors differ with products, it may be rational to select indicators on the basis of a product or a group of similar products. Some factors to be considered in selecting a microbial or chemical indicator for a product (or several similar types of products) are:

1. In a good fresh product, it can be present in low numbers (microbial) or absent (chemical).
2. Under normal conditions of storage (temperature, time, packaging), it should increase (microbial or chemical) in quantity to reach a very high level.
3. When spoilage occurs under normal storage conditions, it should be the predominant causative agent (microbial or chemical).
4. It can be detected rapidly (microbial or chemical).
5. It can be used reliably to predict shelf life and spoilage status (microbial or chemical).
6. It should have a good relationship with the sensory criteria of spoilage of the particular product (microbial or chemical).

Different microbial groups and their metabolites (chemicals) have been evaluated for their suitability as indicators of food spoilage. As bacteria are the most predominant microbial group in food spoilage, the effectiveness of some bacteria and metabolites as indicators is briefly discussed. In addition, the effectiveness of testing microbial heat-stable enzymes in predicting shelf life of products susceptible to spoilage by them is also discussed.

II. MICROBIOLOGICAL CRITERIA

A. Enumeration of Colony-Forming Units (CFUs)

Previous discussions indicated that the initial levels and types of spoilage microorganisms differ with the products or, more accurately, with the sources and intrinsic and extrinsic environments of the products. It is rational to select the microorganisms predominantly involved in spoilage of a food (or a food group) as the indicators of spoilage for that food. As an example, refrigerated ground meat during aerobic storage is normally spoiled by Gram-negative psychrotrophic aerobic rods, most importantly by *Pseudomonas* spp. Thus, the population level of psychrotrophic Gram-negative rods should be the most appropriate indicator of spoilage for this product (or for raw meats stored under the same conditions), both for predicting shelf life of the product and estimating the status of spoilage during storage. Aerobic plate count (APC), which measures mesophilic populations, may not be a good indicator for this product as many mesophiles do not multiply at psychrotrophic temperature and, conversely, some psychrotrophic bacteria do not multiply at 35°C in the 2 d used to enumerate APCs in meats. However, APC (also standard plate count or SPC for dairy products) has special importance in food microbiology. In fresh products, it indicates the effectiveness of sanitary procedures used during processing and handling and before storage of the product. A high APC or SPC in a food product such as chicken salad or pasteurized milk is viewed with suspicion, both for stability and safety. Thus, it is good to include APC or SPC along with the method suitable to detect the load of an appropriate spoilage indicator group for a food, based on its specific type and storage conditions.

Some of the specific microbial groups that can be used as spoilage indicators in different foods (or food types) are listed here. Details of the procedure can be found in books on microbiological examination of foods (also see Appendix E).

1. *Refrigerated Raw (Fresh) Meats Stored Aerobically.* Enumeration of CFUs/g or /cm^2 of psychrotrophic aerobes, especially Gram-negative aerobes. Data can be available in 2 to 7 d, depending on the agar media and indicator, plating methods (pour or surface), and incubation temperature (10 to 25°C) used.
2. *Refrigerated Raw (Fresh) Meats Stored Anaerobically (Vacuum Packaged).* Enumeration of CFUs/g or /cm^2 of psychrotrophic lactic acid bacteria (by plating in a suitable agar medium adjusted to pH 5.0 with lactic acid) as well as psychrotrophic *Enterobacteriaceae* (in violet red bile glucose agar medium) and probably Gram-negative bacteria. Depending on incubation temperature, data can be available in 2 to 7 d. The plates may be incubated in a CO_2 environment for lactic acid bacteria. The products can also be tested for psychrotrophic *Clostridium* spp., such as *Clo. laramie*, by specific methods (strict anaerobic conditions).
3. *Refrigerated Low-Heat-Processed Vacuum-Packaged Meat Products.* Enumeration of CFUs/g or /cm^2 of psychrotrophic lactic acid bacteria (by plating in an agar medium adjusted to pH 5.0) as well as psychrotrophic *Enterobacteriaceae* (in violet red bile glucose agar medium) and Gram-negative bacteria. Depending

on incubation temperature, data can be available in 2 to 7 d. The plates for lactic acid bacteria can be incubated in a CO_2 environment. The products can be tested for psychrotrophic *Clostridium* spp., such as *Clostridium laramie*, by specific methods.

4. *Raw Milk.* SPC, psychrotrophic Gram-negative bacteria, thermoduric bacteria.
5. *Pasteurized Milk.* SPC, psychrotrophic bacteria (Gram-negative and Gram-positive).
6. *Butter.* Lipolytic microorganisms.
7. *Cottage Cheese.* Psychrotrophic, especially Gram-negative bacteria.
8. *Fishery Products (Raw).* Psychrotrophic Gram-negative bacteria.
9. *Beverages.* Aciduric bacteria, yeasts, and molds.
10. *Salad Dressing and Mayonnaise*. *Lactobacillus spp.* (especially *Lactobacillus fructivorans*) and yeasts.

The major disadvantage of microbiological enumeration methods is that it takes several days for population levels of the indicator microorganisms from enumeration of CFUs to become available. To overcome this problem, several indirect methods that indicate the probable population of microorganisms in foods have been devised. One such method is to determine lipopolysaccharides (LPS) present in a food. LPS is specifically found in Gram-negative bacteria. Thus, by measuring LPS concentration (with proper standard curve), the level of Gram-negative bacteria in a food can be estimated. However, this method is not applicable for spoilage by Gram-positive bacteria. Several other indirect methods studied are measurement of ATP (ATP concentrations increase with high numbers of viable cells), impedance or conductivity (electric conductivity decreases with increase in cell numbers), and dye reduction time (higher the population, faster the reduction). For each method, appropriate standard curves are used to determine bacterial levels. However, each method has specific advantages and disadvantages. Also, all methods are not applicable in all food systems (see Appendix E).

B. Phase-Contrast Microscopy

Small and easy-to-use phase-contrast microscopes are available to rapidly identify microbial types (morphology, motility, spore, cell arrangement) present in a food (see cover page). However, the population has to reach to a fairly high level ($\cong 10^{5-6}$/ml) before it can be viewed under a phase-contrast microscope. Also, food particles can interfere with the identification. With practice, it can be a very quick and easy method to get initial ideas about the predominant microbial types (Chapter 20). It is also possible to do quick and direct enumeration of cells by a suitable counting device (e.g., Petroff Hauser counter). The results can be interpreted in several ways. If a desired level is set (specification level), such as spoilage-detection level, one can interpret the result as less than the level (desirable), very close to the level (should be used immediately), or above the level (and disposed). For a nonliquid food, a known amount of food can be suspended in sterile water in a 1:1 dilution, mixed well, and 1 to 2μl of supernatant fluid used on a microscopic slide or a counter for viewing or counting.

III. CHEMICAL CRITERIA

As microorganisms (particularly bacteria) grow in foods, they produce many types of metabolic by-products associated with the spoilage characteristics. If a method is developed that is sensitive enough to measure a specific metabolite in very low concentrations and long before spoilage becomes obvious, then the results can be used to determine the spoilage status of a food. Methods studied thus far to measure microbial metabolites include H_2S production, NH_3 production by colorimetric or titration methods, production of volatile reducing substances, CO_2 production, diacetyl and acetoin production, and indole production. However, different metabolites are produced by different species and strains of bacteria and the results are not consistent; they cannot be used for different types of products.

Change in food pH, especially in meat and meat products, due to microbial growth has also been used to determine the spoilage status of a food. In normal meats, with a pH of ca. 5.5, metabolism of amino acids by some spoilage bacteria generates NH_3, amines, and other basic compounds. This shifts the pH to the basic side (as high as pH 8.0). In contrast, metabolism of carbohydrates (present or added) by some bacteria produces acids and reduces the pH further to the acidic side. Thus, measurement of pH of a stored meat product can also give some indication of its type of spoilage bacteria and the spoilage status (see Table 20.3). As the pH increases, the proteins become more hydrated, i.e., the water-holding capacity (WHC) increases, and, when pressed, this meat has less extract-release volume (ERV); in contrast, when the pH shifts toward the acidic side, the WHC is lower and ERV is higher. However, many low-fat processed meat products are formulated with high phosphate and generally have a pH close to 7.0 (to increase WHC). The buffering action of phosphate may not allow pH to shift to the basic or acidic side from the microbial metabolism of amino acids and carbohydrates, respectively. In these products, pH measurement (or WHC or ERV measurements) may not be good indicators of spoilage status.

None of the microbiological and chemical criteria studied fulfills all the factors necessary for a good indicator that will indicate the expected shelf life of a fresh product as well as its spoilage status during storage. More emphasis needs to be given to develop suitable indicators to reduce the loss of food by microbial spoilage. In the future, biosensors may be developed that could be effective for indicating changes in specific metabolites by a group of bacteria with similar characteristics, that are considered important spoilage bacteria in a food group.

IV. ASSAY OF HEAT-STABLE ENZYMES

A. Heat-Stable Proteinases in Milk

Proteinases of some psychrotrophic bacteria, such as *Pseudomonas fluorescens* strain B52, even when present as low as 1 ng/ml raw milk, can reduce the acceptance quality of UHT-treated milk during normal storage.[5] Because of this, it is very important that sensitive assay methods be used in their estimation to predict the

shelf life of dairy products. Some of the earlier methods, such as UV absorbance, Folin–Ciocalteu reagent reaction, and gel diffusion assay, are probably not sensitive enough for this purpose. Several new methods, such as the use of TNBS (trinitrobenzene sulfonic acid) and fluorescamine reagents are quite sensitive and are being tested to assay proteinases in milk. In the TNBS method, the reagent reacts with free amino groups and, under the experimental conditions, develops color that can be colorimetrically measured to determine the amount of free amino acids present because of proteolysis. Fluorescamine reacts with amino acids to form fluorescent compounds at pH 9.0 and can thus be fluorimetrically measured to determine protein hydrolysis. Other methods, such as enzyme-linked immunosorbant assay (ELISA) and luciferase inactivation assay are extremely sensitive and need further development before they can be used reliably.

B. Heat-Stable Lipases in Milk

Because natural lipases are present in milk, the measurement of lipases produced specifically by psychrotrophic bacteria creates some difficulties.[6] However, it can be overcome by heating the milk, which destroys milk lipases but not the bacterial heat-stable lipases. Assay methods that measure release of free fatty acids (FFAs) due to hydrolysis of milk fat by the lipases can be titrated to determine the potential of lipolysis of the lipases. As milk contains FFAs naturally, this method may not be accurate. Methods in which esterases of chromogenic and fluorogenic compounds react with lipases to produce color or fluorescent products have also been developed, but they have limitations. Recently, a rapid and sensitive sandwich ELISA method was tested to determine lipases of *Pseudomonas* spp. An antibody produced against a *Pse. fluorescens* strain and linked to horseradish peroxidases reacted with lipases from many strains of *Pseudomonas* spp. Reliability and sensitivity of this technique are being studied.

V. CONCLUSION

Food spoilage causes not only economic loss but also unavailability of food, especially in places of food shortage and with high population, such as in some developing countries. To reduce both, efficient techniques need to predict storage potential of foods, especially because the consumption of processed foods is going to increase. The methods now used have limitations and are not highly efficient. New methods that are now being studied could be more effective in the future. One of these methods is the development of biosensors, which could detect important bacterial metabolites at very low concentrations. Another method is to determine bacterial load rapidly by PCR. However, this technique cannot differentiate between live and dead cells. As with lactic acid bacteria at present, if in the future the genome sequences of important spoilage bacteria become available, better and specific biosensors and PCR techniques can be developed to detect spoilage potential of a food at an early stage.

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QUESTIONS

1. List the factors to be considered in selecting indicators of food spoilage.
2. List two spoilage aspects that a suitable food spoilage indicator should indicate to prevent loss of a food by spoilage. Explain why different indicators need to be selected for different groups of foods.
3. Suggest and justify suitable microbial spoilage indicators for (a) tray-packed raw sausage; (b) vacuum-packaged, low-fat, refrigerated ground turkey; (c) fish fillet kept over ice in air; (d) salsa in a bottle stored at room temperature; (e) ready-to-eat baby carrots, ready-to-eat salads, and sprouts of seeds packaged aerobically in bags and stored at ca. 15°C; and (f) cut fruits in aerobically packaged containers at 15°C (consult Chapter 19).
4. List four chemical methods that can be used to detect spoilage of fresh meat stored at refrigerated temperature under aerobic conditions.
5. Explain how pH changes in fresh meats can be used as an indicator of spoilage. How is it related to WHC and ERV?
6. What promises does genome-based nanotechnology hold for the future to detect microbial spoilage potential of a food?

SECTION V

Microbial Foodborne Diseases

Human illness caused by consumption of foods contaminated with factors other than poisons or chemical toxic agents was recognized long before understanding the role of pathogens in foodborne diseases. Dietary guidelines in ancient civilizations, such as eating foods after cooking well and serving warm, and not eating spoiled foods, were probably invoked to protect people at least partly from the danger of microbial foodborne diseases. In the Middle Ages, several mass food poisoning incidents in Europe due to the consumption of grains infested with toxin-producing fungi were recorded. Concern about the possible role of foods of animal origin in human diseases led to the introduction of proper hygienic methods in handling fresh meat. However, not until Pasteur's discovery of the role of microorganisms in foods was their involvement in foodborne diseases understood. This helped search for and isolate pathogens by suitable techniques from foods incriminated in foodborne diseases. *Salmonella* and *Staphylococcus aureus*, because of the high incidence of salmonellosis and staphylococcal poisoning, respectively, and *Clostridium botulinum*, because of the high fatality rate from botulism, were isolated from foods incriminated with foodborne diseases before the 20th century (Chapter 1). Subsequently, many other pathogenic bacteria, toxin-producing molds, and pathogenic viruses were recognized as causative agents in human foodborne illnesses. The involvement of a few new pathogenic bacteria and viruses has been recognized only relatively recently. Even now, the exact role of many bacteria and viruses in foodborne diseases is not well understood, and several of them are designated as opportunistic pathogens. The changes in food production, processing, marketing, and consumption, together with our knowledge about the characteristics of these microorganisms and the development of efficient techniques for their detection, have enabled us to identify the role of some new pathogens in foodborne diseases. History suggests there will probably always be new pathogens, and thus, as we develop methods to control the existing pathogens, we have to remain alert for the new ones. In this section, an overview of the microorganisms associated with foodborne diseases is presented. The diseases caused by the foodborne pathogens have been arbitrarily divided into three groups; the basis of division is not always clear-cut. Also included are some parasites and algae that can cause foodborne diseases. The aspects of new foodborne pathogens and the importance of indicator bacteria on

establishing sanitary quality in food production have been included. The following topics are discussed in this section.

Chapter 23: Important Facts in Foodborne Diseases
Chapter 24: Foodborne Intoxications
Chapter 25: Foodborne Infections
Chapter 26: Foodborne Toxicoinfections
Chapter 27: Opportunist Pathogens, Parasites, and Algal Toxins
Chapter 28: New and Emerging Foodborne Pathogens
Chapter 29: Indicators of Bacterial Pathogens

CHAPTER 23

Important Facts in Foodborne Diseases

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I. INTRODUCTION

The objective of this topic is to recognize the causes of foodborne diseases, the role of microorganisms and several other agents in foodborne diseases, and the importance of predisposing factors in the occurrence of a foodborne disease. This information will help not only understand the epidemiology of foodborne diseases but also develop means of controlling them.

II. HUMAN GASTROINTESTINAL DISORDERS

The causes of foodborne gastrointestinal disorders can be broadly divided into three groups:[1]

1. From consumption of food and water containing viable pathogenic microorganisms or their preformed toxins.
2. From ingestion of pathogenic algae, parasites, and their preformed toxins through food.
3. For reasons other than viable pathogens or their toxins.

Some of the factors included in Group 3 are:

1. Ingestion of toxins naturally present in many foods. This includes certain mushrooms, some fruits and vegetables, and some seafoods.
2. Toxins formed in some foods. Examples are some biological amines (e.g., histamine) that form in some fish, cheeses, and fermented meat products.
3. The presence of toxic chemicals in contaminated food and water, such as heavy metals and some pesticides.
4. Allergy to some normal components of a food. Some individuals are allergic to gluten in cereals and develop digestive disorders following consumption of food containing gluten.
5. Genetic inability to metabolize normal food components. The inability of some individuals to hydrolyze lactose in the small intestine, because of the lack of production of the enzyme lactase, results in digestive disorders (lactose intolerance).
6. Nutritional disorders such as rickets, which is caused by calcium deficiency.
7. Indigestion from overeating or other reasons.

The incidence of foodborne diseases of microbial origin is higher than that for all others causes combined. In the U.S., between 1972 and 1978, among the total number of cases reported of foodborne disease outbreaks, pathogenic microorganisms caused 94.4%, and only 1.1% and 4.3% were caused by parasites and chemicals, respectively. Even at present, the occurrences of foodborne diseases caused by pathogenic microorganisms far exceed those by all others combined. In recent years, foodborne diseases of microbial origin have become the primary food safety concern among U.S. consumers and regulatory agencies. This trend is probably true in most other developed and developing countries.[1]

III. EPIDEMIOLOGICAL ASPECTS

A. Investigation of a Foodborne Disease

In general, various regulatory agencies at the local, state, and national levels are empowered with the responsibility of investigating the cause of a reported foodborne disease (see Appendix C). In the U.S., the regulatory agencies involved in the investigation of a foodborne disease are the local health department; state health, food, and agriculture departments; the federal Food and Drug Administration (FDA); U.S. Department of Agriculture (USDA); Centers for Disease Control and Prevention (CDC); and several others. Initially, a medical doctor who suspects a patient to have a foodborne disease informs the local or state health officials about the incident. These agencies, following a preliminary investigation and recognizing the cause to be of food origin, report the incident to the appropriate federal agencies, which then conduct an epidemiological investigation. The investigation at the state and federal levels involves examination of the suspected foods, environmental samples, and materials obtained from the patients for pathogens, microbial and nonmicrobial toxins, and chemicals. Results of these tests provide direct evidence of the association of an agent (e.g., pathogen, toxin, parasite, chemicals) with the disease. In addition to testing suspected samples, both the sick and other people who consumed the same food from the same source are interviewed to establish an indirect association of the most likely foods with the disease. This information is collected, recorded, and reported by the CDC, Atlanta, GA. (See Section IV of this chapter.)

B. Foodborne Disease Outbreak

In the U.S., the federal regulatory agencies define a foodborne disease as an outbreak when two or more people become sick with a similar illness (symptoms) from the consumption of the same food from the same source, and the epidemiological investigations implicate, either directly or indirectly, the same food from the same source as the cause of the illness. However, in the case of botulism, because of a high fatality rate, even when only one person has the illness, it is considered an outbreak. For chemical poisoning too, even a single case is considered an outbreak.

C. Incidence of Foodborne Disease Outbreak

The incidence of foodborne illnesses in most of the developed countries is lower than in many developing countries.[1–3] The major reasons for the low incidence are the implementation of necessary regulations in production and handling of foods, good sanitary practices, and the availability of necessary facilities to reduce abuse. In the U.S., from 1983 to 1987, an average of 479 foodborne disease outbreaks were reported each year, involving 18,336 individuals (Table 23.1). However, on average, only 38% of the outbreaks, involving ca. 10,908 individuals, were confirmed.[2] This shows that even for the reported incidents, only some could be confirmed from the direct evidence. It is suspected that even in the developed countries, only a small fraction of the total incidents are reported. In many instances, an individual does not consult a doctor, and even if a person does consult a doctor, the incident might not be reported to the regulatory agencies. Based on the mechanisms involved in the surveillance system, it is estimated that in the U.S., ca. 5 million individuals are affected by food- and waterborne diseases every year. Others consider this a conservative estimate. According to them, if, on an average, a person is affected once in 10 years, 10% of the population can become sick annually by foodborne illnesses. In the U.S., this is equivalent to ca. 28 million people annually. Other groups think that the numbers could go as high as 80 million annually. (See Section IV in this chapter.)

D. Cost of Foodborne Diseases

Foodborne illnesses can be fatal as well as cause suffering, discomfort, and debilitation among the survivors. The economic losses from various factors, such as medical treatment, lawsuits, lost wages and productivity, loss of business, recall and destruction of products, and investigation of the outbreaks, can be very high. In the U.S., the annual cost of foodborne diseases is estimated to be more than $20 billion. Costs in 1996 were estimated to be $20 to $37 billion from foodborne illnesses from seven pathogens (*Campylobacter jejuni, Clostridium perfringens, Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella*, *Staphlococcus aureus,* and *Toxoplasma gondii*).[4]

Table 23.1 Foodborne Disease Outbreaks from 1983 to 1987 in the U.S.

	1983	1984	1985	1986	1987	Average/ Year
Reported outbreaks	505	543	495	467	387	479 (100%)
Total cases	14,898	16,420	31,079	12,781	16,500	18,336 (100%)
Confirmed outbreaks	187	185	220	181	136	182 (38%)
Confirmed cases	7,904	8,193	22,987	5,804	9,652	10,908 (59.5%)

E. Predominant Etiological Agents

As indicated before, gastrointestinal disorders can be caused by the consumption of food and water containing pathogenic microorganisms and their toxins; pathogenic algae and parasites and their toxins; toxic chemicals, either natural or as a contaminant; and by other factors. Among these, the largest number of outbreaks, the total number of cases, and the number of deaths are caused by pathogenic bacteria and their toxins. Data presented in Table 23.2 show that in the U.S., from 1983 to 1987, bacteria caused ca. 66% of the outbreaks affecting 92.2% of the cases and 96.4% of the fatalities.[1–3] Outbreaks caused by viruses, parasites, and chemicals were 4.5, 4.0, and 25.5%, respectively. The number of cases and deaths from these agents were also low. No outbreak from molds was reported during this period. Results of a similar U.S. study between 1972 and 1978 showed that percentages of foodborne disease outbreaks and cases, respectively, were as follows: bacterial, 66.3% and 90.9%; viral, 2.7% and 3.5%; parasitic, 7.8% and 1.1%, and chemical 23.5% and 4.3%. This report did not include the number of fatalities. Both reports indicated that pathogenic bacteria are the major cause of foodborne diseases in the U.S. This is probably also true for other countries. Current information for recent years, presented later, also shows the same trend (see Table 23.10). Several factors might be responsible for the high incidence caused by pathogenic bacteria: many pathogenic bacteria are found in the raw food materials of animal and plant origin, many are present in the food environments, many grow very effectively in different foods, and many are not killed by the conditions used for processing of different foods.

F. Types of Microbial Foodborne Diseases

Foodborne diseases in humans result from the consumption of either food and water contaminated with viable pathogenic bacterial cells (or spores in the case of infant botulism) or food containing toxins produced by toxigenic bacteria and molds. On the basis of mode of illnesses, these can be arbitrarily divided into three groups: intoxication or poisoning, infection, and toxicoinfection (Table 23.3).

Table 23.2 Confirmed Foodborne Disease Outbreaks, Cases, and Deaths by Etiological Agents from 1983 to 1987 in the U.S.

Etiological Agents[a]	Outbreaks		Cases		Deaths	
	No.	%	No.	%	No.	%
Bacterial	600	66.0	50,304	92.2	132	96.4
Viral	41	4.5	2,789	5.1	1	0.7
Parasitic[b]	36	4.0	203	0.4	1	0.7
Chemicals[c]	232	25.5	1,244	2.3	3	2.2
Total	909	100	54,540	100	137	100

[a]No incidence from mycotoxins was reported.
[b]Includes *Trichinella spiralis* and Giardia.
[c]Includes Ciguatoxin, scombrotoxin, mushrooms, heavy metals, and other chemicals.

Table 23.3 Microbial Foodborne Diseases and Causative Pathogens

Type of Disease	Causative Microorganism	Microbial Group	Major Symptom Type
Intoxication			
Staph poisoning	*Sta. aureus* strains	Bacteria, Gm+[a]	Gastric
Botulism	*Clo. botulinum* strains	Bacteria, Gm+	Nongastric
Mycotoxin poisoning	Mycotoxin-producing mold strains, e.g., *Asp. flavus*	Molds	Nongastric
Infection			
Salmonellosis	Over 2000 *Salmonella* serovars (except *Sal. typhi* and *Sal. paratyphi*)	Bacteria, Gm–[a]	Gastric
Campylobacter enteritis	*Campylobacter jejuni* and *Cam. coli* strains	Bacteria, Gm–	Gastric
Yersiniosis	Pathogenic strains of *Yer. enterocolitica*	Bacteria, Gm–	Gastric
Enterohemorrhagic *Escherchia coli* colitis	*Esc. coli* 0157:H7	Bacteria, Gm–	Gastric and nongastric
Nonhemorrhagic *Escherchia coli* colitis	Shiga-like toxin (verotoxin) producing *Esc. coli* strains such as *Esc. coli* 026:H11	Bacteria, Gm–	Gastric
Listeriosis	*Lis. monocytogenes* (pathogenic strains)	Bacteria, Gm+	Gastric and nongastric
Shigellosis	Four *Shigella* species (e.g., *Shi. dysenterie*)	Bacteria, Gm–	Gastric
Vibrio parahaemolyticus gastroenteritis	Pathogenic strains of *Vib. parahaemolyticus*	Bacteria, Gm–	Gastric
Vibrio vulnificus infection	*Vib.vulnificus* strains	Bacteria, Gm–	Gastric and nongastric
Brucellosis	Brucella abortus	Bacteria, Gm–	Gastric and nongastric
Viral infections	Pathogenic enteric viruses (e.g., Hepatitis A virus)	Viruses	Gastric and nongastric
Toxicoinfection			
Clostridium perfringens gastroenteritis	*Clo. perfringens* strains	Bacteria, Gm+	Gastric
Bacillus cereus gastroenteritis	*Bac. cereus* strains	Bacteria, Gm+	Gastric
Escherchia coli gastroenteritis	Enteropathogenic and enterotoxigenic *Esc. coli* strains		
	Bacteria, Gm–	Gastric	
Cholera	Pathogenic strains of *Vib. cholerae*	Bacteria, Gm–	Gastric
Gastroenteritis by Opportunist Pathogens			
Aeromonas hydrophila gastroenteritis	*Aer. hydrophila* strains	Bacteria, Gm–	Gastric
Plesiomonas shigelloides gastroenteritis	*Ple. shigelloides* strains	Bacteria, Gm–	Gastric

[a] Gm+, Gm–: Gram-positive and -negative, respectively.

1. Intoxication

Illness occurs as a consequence of ingesting a preformed bacterial or mold toxin because of its growth in a food. A toxin has to be present in the contaminated food. Once the microorganisms have grown and produced toxin in a food, there is no need of viable cells during consumption of the food for illness to occur. Staph food poisoning is an example.

2. Infection

Illness occurs as a result of the consumption of food and water contaminated with enteropathogenic bacteria or viruses. It is necessary that the cells of enteropathogenic bacteria and viruses remain alive in the food or water during consumption. Viable cells, even if present in small numbers, have the potential to establish and multiply in the digestive tract to cause the illness. Salmonellosis and hepatitis A are examples.

3. Toxicoinfection

Illness occurs from ingesting a large number of viable cells of some pathogenic bacteria through contaminated food and water. Generally, the bacterial cells either sporulate or die and release toxins to produce the symptoms. *Clo. perfringens* gastroenteritis is an example.

In addition to the pathogenic microorganisms associated with foodborne illnesses, some bacterial species and strains normally considered nonpathogenic can cause gastroenteritis, especially in susceptible individuals. They are designated as opportunistic pathogens. They are normally required to be alive and present in large numbers when consumed through a contaminated food.

G. Predominant Bacterial and Viral Pathogens Associated with Foodborne Diseases

Although many pathogenic bacterial species and viruses have been implicated with foodborne (and waterborne) disease outbreaks, some have occurred at a higher frequency than others (Table 23.4). Of the two most common pathogens associated with foodborne intoxication from 1983 to 1987, the number of deaths was higher for *Clo. botulinum*, but the total number of cases was much higher for *Sta. aureus*. Among the enteric pathogens, the largest number of outbreaks, cases, and fatalities resulted from foodborne infections caused by *Salmonella*. Toxicoinfection outbreaks and number of cases were higher for *Clo. perfringens* than for *Bacillus cereus*. Of the two most common viral diseases from contaminated food and water, the number of outbreaks was higher for hepatitis A, but Norwalk-like viruses affected more people. From 1983 to 1987, salmonellosis was associated with the highest number of outbreaks of all foodborne diseases, affecting the largest number of individuals and causing the most deaths. In contrast, botulism affected the least number of people but caused the highest number of deaths in the affected people (17 out of 140). No death was reported from staphylococcal intoxication, *Bac. cereus* gastroenteritis, or

Table 23.4 Predominant Bacterial and Viral Pathogens Associated with the Confirmed Foodborne Diseases from 1983 to 1987 in the U.S.

Bacteria and Viruses[a]	Outbreaks		Cases		Deaths	
	No.	%	No.	%	No.	%
Staphylococcus aureus	47	7.6	3,181	6.2	0	0
Clostridium botulinum	74	11.9	140	0.3	10	17.0
Salmonella	342	55.1	31,245	61.1	39	66.0
Shigella spp.	44	7.1	9,971	19.5	2	3.4
Escherichia coli	7	1.1	640	1.3	4	6.8
Campylobacter spp.	28	4.5	727	1.4	1	1.7
Clostridium perfringens	24	3.9	2,743	5.4	2	3.4
Bacillus cereus	16	2.6	261	0.5	0	0
Hepatitis A virus	29	4.7	1,067	2.0	1	1.7
Norwalk-like virus	10	1.5	1,164	2.3	0	0
Total	621	100	51,139	100	59	100

[a]Not included in the table are *Brucella* spp., *Streptococcus* spp., *Vibrio* spp., and several others, which combined were associated with 18 (2.8%) outbreaks. *Lis. monocytogenes* caused 3 outbreaks affecting 259 people, with 70 deaths. See Table 23.11 for current trends.

Norwalk-like viral infection from 1983 to 1987. Some reasons for higher incidence of some pathogens, such as *Salmonella*, over others can be their occurrence in higher frequency in the food environments, their greater ability to produce the disease, and their ability to grow more rapidly under abusive conditions. With some pathogens, a single outbreak can involve many cases. This is typical for salmonellosis, shigellosis, and staphylococcal intoxication. For other pathogens, such as *Clo. botulinum*, only a few individuals are affected in a single outbreak. This is because of the predisposing factors associated with a particular outbreak. This aspect is explained later with the specific disease. *Lis. monocytogenes*, not included in this data, caused 70 deaths among 259 cases in 3 outbreaks.[2]

H. Predominant Food Types Associated with Foodborne Diseases of Bacterial and Viral Origin

Certain food types or foods prepared under specific conditions and environments have been implicated more frequently with foodborne disease than others. Some of the factors could be the presence of a pathogen in the raw materials in higher frequency, thereby having a greater chance to contaminate the finished products, the ability of a pathogen to grow advantageously in a particular type of food, a greater chance of failure in quality control in a specific environment, and a higher possibility of food handlers to contaminate the finished products. Some of these aspects can

be explained from the foodborne disease outbreak data presented in Table 23.5. Foods of animal origin (meat, fish, eggs, and dairy products) were implicated in 23% of the outbreaks, and *Salmonella* serovars were involved in large proportions (except for fish); the latter may arise because many food animals harbor *Salmonella* as carriers in the digestive tract and can thus contaminate meat, eggs, and dairy products. In fish products, the incidence of botulism outbreaks was high, because they can be contaminated with *Clo. botulinum* (type: nonproteolytic E) present in some marine environments. In recent years, fish consumption has increased and so have the foodborne disease outbreaks from fishery products. Similarly, fruits and vegetables can be contaminated with *Clo. botulinum* from the soil. In contrast, salads, which are handled extensively, can be contaminated with several pathogens of human origin. The high incidence of *Clo. perfringens* in Mexican foods and *Bac. cereus* in Chinese fried rice is likely due to the methods used in the preparation of certain ethnic foods. The food types in 40% of the outbreaks could not be determined, because the food samples were not available for testing in many cases. However, one can speculate from the high incidence of *Salmonella* in both multiple food and unknown food categories that many were probably foods of animal origin.[2]

Table 23.5 Predominant Food Types Associated with Confirmed Foodborne Disease Outbreaks of Bacterial and Viral Origin from 1983 to 1988 in the U.S.

Food Types	No. of Outbreaks	%	Predominant Pathogens (% No. of Outbreaks)
Meat products[a]	91	14.0	*Salmonella* (53%), next *Sta. aureus*
Fish products[b]	20	3.0	*Clo. botulinum* (50%)
Egg products	11	2.0	*Salmonella* (82%)
Dairy products	26	4.0	*Salmonella* (27%)
Salads[c]	33	5.0	*Salmonella, Sta. aureus, Shigella* spp.
Baked food	0	1.0	*Sta. aureus*
Fruits and vegetables	44	7.0	*Clo. botulinum*
Mushrooms	2	0.5	*Clo. botulinum*
Beverages	3	0.5	*Salmonella*
Ethnic foods[d]	19	3.0	*Clo. perfringens, Bac. cereus, Salmonella*
Multiple foods[e]	123	19.0	*Salmonella* (59%)
Unknown foods[f]	254	40.0	*Salmonella* (68%), *Shigella* spp., viruses

[a] Includes beef, ham, pork, sausages, chicken, turkey, and stews.
[b] Includes shellfish and fin fish.
[c] Includes potato, chicken, fish, egg, and other salads.
[d] Includes fried rice, Chinese food, and Mexican food.
[e] More than one food was involved in an outbreak.
[f] Although food was implicated with an outbreak, involvement of a food was not confirmed.

I. Predominant Places of Food Consumption Associated with Confirmed Foodborne Disease Outbreaks of Bacterial and Viral Origin

An analysis of the relationship between the places of food consumption and the number of foodborne disease outbreaks revealed that, at least in the U.S., the highest number of incidents occurred with foods served at food establishments (Table 23.6). These include fast-food services, restaurants, cafeterias, and schools. Several factors can be associated with this.[2] The total number of meals served is very high. Also, high proportions of food are served within a very short period of time. In addition, the number of people involved in handling the food, many of whom may not have training in safe food handling, is very high. This can result in a greater chance of contamination and failure to observe proper sanitation. Improper cooling of foods, cross-contamination, and contamination of food from pets can be the reasons for foodborne diseases with foods served in homes. Mishandling of foods, temperature abuse, long intervals between preparation and consumption, and improper sanitation can be associated with outbreaks at picnics. The exact sources could not be identified in 30% of the outbreaks; from the available information, it is most likely that many of them were probably with foods served at food establishments. Although not listed in Table 23.6, the relative frequency of foodborne disease outbreaks with foods from large commercial processors is low. This is generally because they use good quality control, sanitation, and testing of the products for the pathogens. However, when a contamination of food by a pathogen occurs in a big processing establishment, a large number of people over a wide area are affected.[2]

J. Predominant Contributing Factors Associated with Confirmed Foodborne Disease Outbreaks from Pathogenic Bacteria and Viruses

Before foods are consumed, they are exposed to many different environments and conditions. These dictate whether a pathogen present initially will survive or be

Table 23.6 Predominant Establishments of Food Consumption Associated with Confirmed Foodborne Disease Outbreaks from Pathogenic Bacteria and Viruses from 1983 to 1987 in the U.S.

Establishment	No. of Outbreaks	%
Homes	149	24.8
Food services[a]	225	37.5
Picnic[b]	45	7.5
Processing plants[c]	NA	NA
Unknown[d]	181	30.2
Total	600	100

[a] Includes fast-food establishments, restaurants, cafeterias, and school lunch programs.
[b] Includes family picnics, church picnics, and camps.
[c] Data not available.
[d] Exact places not identified.

Table 23.7 Predominant Contributing Factors Associated with Confirmed Foodborne Disease Outbreaks from Pathogenic Bacteria and Viruses from 1983 to 1987 in the U.S.

Contributing Factor	1983	1984	1985	1986	1987	Total (%)
Improper holding temperature	52	68	57	40	27	244 (34.6)
Poor personal hygiene	21	42	38	25	19	145 (20.5)
Inadequate cooking	24	19	36	20	19	118 (16.7)
Contaminated equipment	17	33	25	21	12	108 (15.3)
Food from unsafe sources	15	3	10	5	6	39 (5.5)
Others	9	12	12	11	8	52 (7.4)

killed, if recontamination can occur, or if a pathogen can multiply to reach a high population to cause disease. The foodborne disease outbreak data between 1983 and 1987 in the U.S. revealed that the most predominant cause of the outbreaks was improper holding temperature (refrigerated temperature) of the food (Table 23.7; also compare Table 23.12 for current trends). The temperature abuse resulted in the growth of pathogens (bacteria) to reach to a level that causes illness following consumption. The other factors, in order of importance, were contamination of foods due to poor personal hygiene, survival of pathogens due to cooking at lower temperatures than specified, cross-contamination of foods from equipment (previously contaminated with pathogens), and food from unsafe sources, such as raw foods. These five factors, in combination, were responsible for 92.6% of the outbreaks caused by pathogenic bacteria and viruses.[2]

K. Influence of Month (of the Year) on Number of Foodborne Disease Outbreaks Caused by Pathogenic Bacteria and Viruses

In general, foodborne outbreaks of pathogenic bacterial and viral origin are more prevalent during the summer months. The compiled data for 1983 to 1987 (Table 23.8) show that in the U.S., ca. 66% of the outbreaks occurred during May to September, with the highest incidence in August.[2] Between November and April, the incidence was reduced to ca. 34%. During commercial processing, food products are exposed to indoor temperature, which may not vary greatly in the summer or the winter. However, both raw products and processed products can be exposed to outside temperatures for a long time during transportation, displays in stores, at food establishments, and at home. Also, during the summer, picnics and outdoor eating

Table 23.8 Influence of the Month of the Year to Confirmed Foodborne Disease Outbreaks from Bacteria and Viruses from 1983 to 1987 in the U.S.

	No. of Outbreaks			
Months	Total (%)	Average	Range	Comments
May to October	413 (65.7)	69	57 to 86	Highest in August
November to April	216 (34.3)	36	26 to 45	Lowest in February

occur more frequently. A high temperature during the summer can stimulate rapid growth of the contaminating pathogens to reach a high level, even from a low initial level, within a relatively shorter period. During the winter months, the growth rate can be greatly reduced.

L. Influence of Location on Foodborne Diseases of Pathogenic Bacterial and Viral Origin

In general, the incidence of foodborne disease outbreaks is lower in the developed countries than in the developing countries. Several socioeconomic reasons and climatic conditions cause this difference. However, the frequency of outbreaks can vary greatly among regions, both in the developed and developing countries. In the U.S., foodborne disease outbreaks have been recorded more frequently in some states than in others. States with high populations, large numbers of industries, and warm climates have more incidents. States in this group are New York, Washington, California, Hawaii, and Pennsylvania (Table 23.9). Some other factors, such as a large ethnic population, greater frequency of migration and traveling of people, as well as better surveillance systems, can also cause a high incidence rate. In comparison, states with lower populations had very few incidents.

M. Human Factors in Foodborne Disease Symptoms

When a group of people consumes food contaminated with live cells of pathogens or their toxins, all the members might not develop disease symptoms.[1,2] Among those who develop symptoms, all might not show either the same symptoms or the same severity of a symptom. This is probably due to the difference in resistance among individuals. One of the factors involved in developing symptoms from the consumption of a contaminated food is susceptibility of an individual to the contaminants. In general, infants and old, sick, and immunodeficient people are more susceptible than normal adults and healthy individuals. The chance of developing disease symptoms is directly related to the amount of a contaminated food consumed. This is related to the number of viable cells of a pathogen or the amount of a toxin consumed by an individual. The virulence of a pathogen or a toxin consumed through a food also determines the onset of a disease and severity of symptoms. For some

Table 23.9 Influence of Location on Confirmed Foodborne Disease Outbreaks from Pathogenic Bacteria and Viruses from 1983 to 1987 in the U.S.

High Incidence			Low Incidence		
State	**Total**	**Range**	**State**	**Total**	**Range**
New York[a]	744	129 to 174	Wyoming	2	0 to 1
Washington	243	38 to 55	Montana	3	0 to 2
California	166	30 to 38	S. Dakota	4	0 to 2
Hawaii	138	20 to 36	Nevada	1	0 to 1
Pennsylvania	96	15 to 24	Utah	6	0 to 1

[a] New York City had 216 outbreaks with a range of 24 to 80 during the same period.

highly virulent pathogens, such as *Esc. coli* O157:H7, consumption of as low as ten viable cells can cause disease in an infant. In contrast, for some pathogens, such as *Yersinia enterocolitica*, consumption of as high as 1 million or more viable cells is necessary for the symptoms to develop.[1–3]

O. Acceptance Quality of Food Because of Growth of Pathogens

Pathogenic viruses need viable host cells for growth; thus they cannot grow in prepared foods, and do not affect the food quality. Pathogenic bacteria can grow in many foods. When the environment is suitable, only a few viable cells present initially can reach a high level, maybe several millions per gram or milliliter. However, growth of some pathogens, even to a high level, may not alter the color, texture, and odor of a food (such as *Sta. aureus*). People can consume this food unknowingly and develop symptoms of a foodborne illness.

P. Sequence of Events in a Foodborne Disease

For a foodborne disease to occur, several events have to happen in sequence (Figure 23.1).[1,2] Understanding these sequences is helpful to investigate the cause (source and means of transmission) of a foodborne disease. It also helps recognize how the sequence can be broken in order to stop a foodborne disease. Initially, there has to be a source of a pathogen. Next, the pathogen has to contaminate a food. Consumption of the food contaminated with a pathogenic virus can lead to viral infection. For bacterial pathogens (and toxicogenic molds) the contaminated food has to support growth and be exposed for a certain period of time at a suitable temperature to enable the pathogens to grow. However, for some potent pathogens (such as *Esc. coli* O157:H7), growth may not be necessary to cause a foodborne infection. For intoxication, growth should reach a sufficient level to produce enough toxin so that when the food is consumed, the individual develops the symptoms. For bacterial infection, viable cells of a pathogen need to be consumed in sufficient numbers, which vary greatly with pathogens, to survive stomach acidity, establish in the digestive tract, and cause illness. For toxicoinfection, viable cells should be consumed either in very high numbers (for those that cannot multiply in the digestive tract, such as *Clo. perfringens*) or in reasonable numbers (for those that can multiply in the digestive tract, such as *Vibrio cholerae*) so that toxins released by them in the digestive tract can produce the symptoms.

IV. CURRENT TRENDS

In 1997, a summary review on foodborne illnesses in the U.S. between 1988 and 1992 was published. At present, through FoodNet, a foodborne disease surveillance network of regulatory agencies has been set up, which makes it possible to obtain available foodborne outbreak reports relatively quickly. In addition, a recent article from the CDC, based on a scientific approach, listed the estimated foodborne illnesses for all currently known foodborne pathogens. The information is summarized

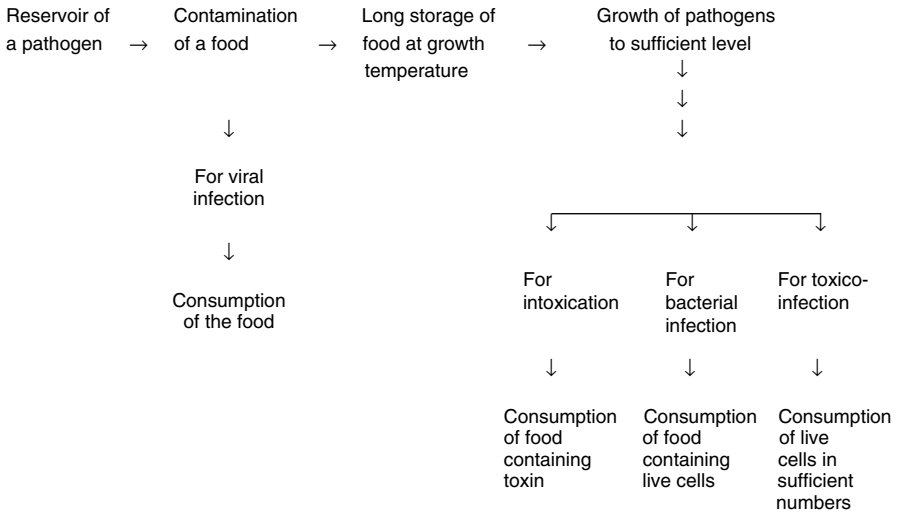

Reservoir of a pathogen
Contamination of a food
Long storage of food at growth temperature
Growth of pathogens to sufficient level
For viral infection
Consumption of the food
For intoxication
For bacterial infection
For toxico-infection
Consumption of food containing toxin
Consumption of food containing live cells
Consumption of live cells in sufficient numbers

Figure 23.1 Sequence of events leading to a foodborne disease by pathogenic bacteria and viruses.

here to evaluate the current trends of foodborne illnesses as compared with those in the past (between 1982 and 1987; presented in tables in this chapter).

A. Foodborne Disease Outbreaks during 1988 to 1992

Bean et al. (1997)[5] reviewed the data collected by the CDC on reported foodborne disease outbreaks in the U.S. during 1988 to 1992. During this period (Table 23.10), as before (Table 23.2), the incidence of outbreaks, number of cases, and number of deaths declined, but were still much higher than other etiological agents. Like the previous period, the incidence of illnesses during this period was highest from foods of animal origin (Table 23.5) and occurred more frequently during May to October, with the highest incidence during June to August (Table 23.8). Similarly, as before (Table 23.6), homes (15.2%) and food services (35.7%) were the major sources of

Table 23.10 Foodborne Disease Outbreaks, Cases, and Deaths by Etiological Agents from 1988 to 1992 in the U.S.

Etiological Agents	Outbreaks		Cases		Deaths[a]	
	No.	%	No.	%	No.	%
Bacterial	796	32.9	33,183	42.9	55	79.7
Viral	45	1.8	2,401	3.1	6	8.7
Parasitic	17	<1.0	379	<1.0	0	0
Chemicals	143	5.9	927	1.2	4	5.8
Unknown	1,422	58.7	40,487	52.3	4	5.8
Total	2,423	≅100	77,377	≅100	69	100

[a]Death: *Clo. botulinum*, 11; *Salmonella*, 38; *Lis. monocytogenes*, 1; *Vib. cholerae*, 1; *Clo. perfringens*, 1; Hepatitis A, 4; paralytic shellfish, 2; scombroid, 1.

Table 23.11 Reported Foodborne Disease Outbreaks from Bacterial and Viral Pathogens from 1988 to 1992 in the U.S.

Pathogens	Outbreaks		Cases	
	No.	%	No.	%
Bac. Cereus	21	2.5	433	1.2
Cam. jejuni	27	3.2	703	2.0
Clo. botulinum	60	7.1	133	<1.0
Clo. perfringens	40	4.8	3801	10.7
Esc. coli O157:H7	11	1.3	244	<1.0
Salmonella[a]	549	65.4	21,177	59.5
Shigella	25	2.9	4,788	13.5
Sta. aureus	50	5.9	1,678	4.7
Lis. monocytogenes	1	<1.0	2	<1.0
Vib. cholerae	4	<1.0	21	<1.0
Vib. parahaemolyticus	4	<1.0	21	<1.0
Vib. vulnificus	1	<1.0	2	<1.0
Streptococcus Group A	2	<1.0	135	<1.0
Hepatitis A	43	5.1	2109	5.9
Norwalk-like virus	2	<1	292	<1.0
Total	840	≅100	35,581	≅100

[a]Serovar Enteritidis was involved in 329 (59.9%) of the total *Salmonella* outbreaks, of which 96 (17.5%) were identified with the consumption of eggs.

foodborne illness during this period. Among the pathogens, the incidence was the highest with *Salmonella*, 65.4%; Table 23.11) and showed an increase of 10% over the previous period (55.1%; Table 23.4). Serovar Enteritidis was the predominant isolate. The frequency of incidence of outbreaks for the other pathogens remained very similar during both periods. During both periods, the three major contributing factors for the outbreaks remained essentially the same, namely, improper holding temperature, poor personal hygiene, and inadequate cooking (Table 23.7 and Table 23.12). The frequency of contaminated equipment (including cross-contamination) increased slightly during this period.

A comparison of the results for the two 5-year periods indicates very little change in the epidemiology of foodborne disease outbreaks in the U.S.

Table 23.12 Contributing Factors Associated with Foodborne Disease Outbreaks from 1988 to 1992 in the U.S

Contributing Factors	Outbreaks	
	No.	%
Improper handling temperature	848	36.7
Poor personal hygiene	554	22.3
Inadequate cooking	401	17.4
Contaminated equipment	229	9.9
Food from unsafe sources	161	7.0
Other and unknown	155	6.8

B. FoodNet

The CDC, along with the Food Safety Inspection Service (FSIS) of the USDA, the FDA, and the eight Emerging Infections Program (EIP) sites developed the FoodNet Program in an effort to reduce foodborne diseases in the U.S. The major objectives of FoodNet are to determine (a) the frequency and severity of foodborne diseases; (b) the proportion of common foodborne diseases that result from eating specific foods; and (c) the epidemiology of new bacterial, parasitic, and viral foodborne pathogens. To obtain the information, FoodNet conducts active surveillance and related epidemiological studies. FoodNet can determine the effectiveness of the programs initiated by an interagency national food safety initiative to reduce foodborne diseases. One example is to study the effectiveness of the USDA Pathogen Reduction and Hazard Analysis Critical Control Point (HACCP) rule in reducing the number of cases of major foodborne diseases in the U.S. each year. FoodNet is a sentinel network that can respond rapidly to new and emerging foodborne pathogens and evaluate new prevention and control strategies to safeguard the food and health of consumers.[6]

FoodNet was established in 1996 by directly linking CDC, USDA-FSIS, and the FDA with the state and local health departments in the states of California, Connecticut, Georgia, Maryland, Minnesota, New York, and Oregon. As of 1999, FoodNet conducted population-based active surveillance for confirmed cases of nine pathogens, namely, *Campylobacter, Salmonella, Shigella, Esc. coli* O157:H7, *Lis. monocytogenes, Yer. enterocolitica, Vibrio* spp., *Cryptosporidium,* and *Cyclospora* (the last two were included since 1997), covering 25.6 million people.[6–9]

Table 23.13 summarizes the findings between 1996 and 1999. Over the 4-year period, the incidence from *Campylobacter, Shigella,* and *Esc. coli* O157:H7 declined. The incidence of *Salmonella* declined in 1997 and 1998, but increased again in 1999.

Table 23.13 Rate of Incidence of Nine Foodborne Diseases from 1996 to 1999 in the U.S.

	Rate Per 100,000 Population[a]			
Pathogen	**1996**	**1997**	**1998**	**1999[b]**
Campylobacter spp.	23.5	25.2	21.4	17.3
Salmonella[c]	14.5	13.6	12.3	14.8
Shigella spp.	8.9	7.5	8.5	5.0
Esc. coli O157:H7	2.7	2.3	2.8	2.1
Lis. monocytogenes	0.5	0.5	0.6	0.5
Yer. enterocolitica`	1.0	0.9	1.0	0.9
Vibrio spp.	0.2	0.3	0.3	0.2
Cryptosporidium	NI[d]	3.0	3.4	2.9
Cyclospora	NI	0.3	<0.1	<0.1

[a] Highest incidence in June to August during 1996 and 1999.
[b] In 1999, among the 10,667 confirmed cases, the incidence of nine diseases were salmonellosis, 4533; campylobacteriosis, 3794; shigellosis, 1031; *Esc. coli* O157:H7 infection, 530; cryptosporidiosis and cyclosporiasis, 14.
[c] Among the *Salmonella* isolates, the predominant serotypes were serovar Typhimurium, 24%, serovar Enteritidis, 10%; serovar Newport, 9%; serovar Heidelberg, 6%, and serovar Muenchen, 6%.
[d] NI: Not included.

The rates from serovar Typhimurium were constant between 1996 and 1999 (ca. 3.7%), but rates for serovar Enteritidis declined. The incidence of *Lis. monocytogenes*, *Vibrio* spp., and *Yer. enterocolitica* remained unchanged during the 4-year period. During the last 3 years, the incidence of *Cyclospora* declined, but that of *Cryptosporadium* remained almost unchanged.[6]

C. Estimated Foodborne Illnesses

For a long time, opinions differed on the actual number of foodborne illness cases vs. the number of cases reported annually in the U.S. The average number of ca. 20,000 cases reported annually was thought to be too low. A realistic figure was estimated to be anywhere from 5 to 25 million, and some thought it to be as high as 80 million.

Recently, a group of researchers published a report on the estimated number of cases of foodborne illnesses from both known and unknown causes.[7] They compiled and analyzed the information gathered by several surveillance systems (e.g., FoodNet) and other relevant sources. According to this report, the total number of foodborne illnesses from known and unknown causes was estimated to be 76 million annually, with 325,000 hospitalizations and 5000 deaths in the U.S. Among these, the known pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1800 deaths (Table 23.14). About 75% of these deaths were caused by *Salmonella, Listeria*, and *Toxoplasma*.

The report indicated that via different surveillance programs, an accurate number of illnesses associated with outbreaks caused by some pathogens was available. However, data for sporadic illnesses, especially for pathogens such as *Bac. cereus, Clo. perfringens,* and *Sta. Aureus*, are not accurately available through such programs. For these pathogens, the outbreak-related cases were multiplied by 10 to obtain the total number of illnesses through sporadic and outbreak incidences. The basis of using this multiplier was from data in which both sporadic and outbreak illnesses were available, such as for *Salmonella* and *Shigella*.

Some other important aspects mentioned in this report are that even with the current surveillance programs, the foodborne illnesses are underreported. any pathogens that are transmitted through food can also be transmitted through water and person to person, thus obscuring the role of foodborne transmission. Also some of the current foodborne illnesses are caused by pathogens that have not yet been identified.[7]

V. CONCLUSION

Foodborne diseases, either sporadic or outbreaks, can be caused by different pathogenic microorganisms, some of which are more predominant than others. The different parameters associated with the predominant factors (pathogenic bacteria and viruses) are also described. Finally, the sequence of events necessary for a foodborne disease to occur is discussed. This information will be helpful in understanding the material presented in following chapters.

Table 23.14 Estimated Yearly Cases of Foodborne Diseases and Related Deaths in the U.S.

Etiological Agent	Illnesses	Deaths
Bacterial		
Bac. cereus	27,360	0
Clo. botulinum	58	4
Brucella spp.	777	6
Campylobacter spp.	1,963,141	99
Clo. perfringens	248,520	7
Esc. coli O157:H7	62,458	52
Esc. coli non-O157:H7	31,299	26
Esc. coli enterotoxigenic	55,594	0
Esc. coli other	23,826	0
Lis. monocytogenes	2,493	499
Sal. typhi	659	3
Salmonella (others)	1,341,873	553
Shigella spp.	89,648	14
Sta. aureus	185,060	2
Streptococcus	50,920	0
Vib. cholerae	49	0
Vib. vulnificus	47	18
*Vibrio (*others)	5,122	13
Yer. enterocolitica	86,731	2
Subtotal	4,175,565	1,297
Viral		
Norwalk-like	9,200,000	124
Rotavirus	39,000	0
Astovirus	39,000	0
Hepatitis A	4,170	4
Subtotal	9,282,170	129
Parasitic		
Cryptosporidium parvum	30,000	7
Cyclospora cayetanensis	14,638	0
Giardia lamblia	200,000	1
Toxoplasma gondii	112,500	375
Trichinella spiralis	52	0
Subtotal	357,190	383
Grand total	13,814,924	1,809

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QUESTIONS

1. List the factors associated with a foodborne disease outbreak.
2. Define or explain an outbreak, a reported case, and a confirmed outbreak.
3. Survey 25 of your friends and relatives about the number of individuals who had, at least once, a digestive disorder in the last 2 years. From this, calculate approximately how many people can be affected with foodborne disease in the U.S. per year. Is it close to 20,000? 5 million? 25 million? Or 80 million?
4. From the survey in Question 3, estimate the most common food type (animal, plant) associated with foodborne diseases.
5. From the survey in Question 4, use one incident and discuss the symptoms, time of occurrence of symptoms after eating, the likely pathogen, probable source of the pathogen, and predisposing factors that caused the disease.
6. Define foodborne intoxication, infection, and toxicoinfection, and give two examples for each.
7. Discuss why pathogenic bacteria are the predominant agents in foodborne diseases.
8. "Foods of animal origin are more frequently associated with foodborne disease outbreaks." Justify or explain the statement.
9. List the possible reasons for higher incidence of foodborne diseases with food served at food service establishments.
10. List, in order of importance, the five most important contributing factors in the occurrence of foodborne disease of pathogenic bacterial and viral origin.
11. List the sequence of events in case of a salmonellosis outbreak.
12. Briefly discuss what you have learned from the material in this section.

CHAPTER 24

Foodborne Intoxications

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I. INTRODUCTION

Foodborne intoxication or food poisoning of microbial origin occurs by ingesting a food containing a preformed toxin. Two of bacterial origin, staphylococcal intoxication and botulism, and mycotoxicosis of mold origin are briefly discussed in this chapter. Although *Bacillus cereus* can also form a heat-stable toxin and produce intoxication, this aspect is discussed under toxicoinfection. The discussions include relative importance of a disease, characteristics of the microorganisms involved, predominant types of food, nature of the toxins, disease and the symptoms, preventative measures, and, for some, analysis of actual outbreak.

Some general characteristics of food poisoning are:

1. The toxin is produced by a pathogen while growing in a food.
2. A toxin can be heat labile or heat stable.
3. Ingestion of a food containing active toxin, not viable microbial cells, is necessary for poisoning (except for infant botulism, in which viable spores need to be ingested).
4. Symptoms generally occur quickly, as early as 30 min after ingestion.
5. Symptoms differ with type of toxin; enterotoxins produce gastric symptoms and neurotoxins produce neurological symptoms.
6. Febrile symptom is not present.

II. STAPHYLOCOCCAL INTOXICATION

A. Importance

Staphylococcal food poisoning (staphylococcal gastroenteritis; staphylococcal food poisoning; staph food poisoning), caused by toxins of *Staphylococcus aureus*, is considered one of the most frequently occurring foodborne diseases worldwide.[1,2] In the U.S., at least before the 1980s, it was implicated in many outbreaks. However, in recent years, the number of staphylococcal food poisoning outbreaks has declined. CDC reports indicate that during 1972 to 1976, it was associated with 21.4% of the foodborne disease outbreaks affecting 29.7% of the total cases; in contrast, between

1983 and 1987, there were 5.2% staphylococcal foodborne outbreaks affecting 5.8% of the cases, with no deaths. This decline is probably a reflection of the better use of refrigerated temperatures to store food and improved sanitary practices that can control contamination and growth of *Sta. aureus*. Even then, the number of outbreaks and number of cases of staphylococcal gastroenteritis is much higher than several other microbial foodborne disease outbreaks.

B. Characteristics of *Staphylococcus aureus*

1. Organisms

Sta. aureus are Gram-positive cocci, occur generally in bunches, and are nonmotile, noncapsular, and nonsporulating.[3–5]

2. Growth

Most strains ferment mannitol and produce coagulase, thermonuclease, and hemolysin, but differ in their sensitivity to bacteriophages. The cells are killed at 66°C in 12 min, and at 72°C in 15 s. *Sta. aureus* are facultative anaerobes, but grow rapidly under aerobic conditions. They can ferment carbohydrates and also cause proteolysis by extracellular proteolytic enzymes. They are mesophiles with a growth temperature range of 7 to 48°C, with fairly rapid growth between 20 and 37°C. They can grow at relatively low A_w (0.86), low pH (4.8), and high salt and sugar concentrations of 15% and in the presence of NO_2. However, their growth can be reduced by combining two or more parameters. Because of their ability to grow under several adverse conditions, *Sta. aureus* can grow in many foods. Normally, they are poor competitors to many other microorganisms found in foods. But their ability to grow under adverse environments gives them an edge in growth in many foods in which others do not grow favorably.[3–5]

3. Habitat

Enterotoxin-producing *Sta. aureus* strains have generally been associated with staphylococcal food intoxication. Although strains of several other *Staphylococcus* species are known to be enterotoxin producers, their involvement in food poisoning is not fully known. *Sta. aureus*, along with many other staphylococci, are naturally present in the nose, throat, skin, and hair (feathers) of healthy humans, animals, and birds. *Sta. aureus* can be present in infections, such as cuts in skin and abscesses in humans, animals, and birds, and cuts in hands and facial-erupted acne in humans. Food contamination generally occurs from these sources.[3–5]

C. Toxins and Toxin Production

Enterotoxigenic strains of *Sta. aureus* produce seven different enterotoxins: A, B, C1, C2, C3, D, and E (also designated as SEA, SEB, etc.).[3,4,6,7] They are serologically distinct heat-stable proteins of molecular weight 26 to 30 kDa and differ in

toxicity. The toxins vary in heat stability, SEB being more stable than SEA. Normal temperature and time used to process or cook foods do not destroy the potency of the toxins. Outbreaks from SEA are more frequent, probably because of its high potency.

Rate of toxin production by a strain is directly related to its rate of growth and cell concentrations. Optimum growth occurs ca. 37 to 40°C. Under optimum conditions of growth, toxins can be detected when a population has reached over a few million per gram or milliliter of food and generally in ca. 4 h. Some of the lowest environmental parameters of toxin production are 10°C, pH 5.0, or A_w 0.86. However, by combining two or more parameters, the lowest ranges can be adversely affected.

D. Disease and Symptoms

Staphylococcal toxins are enteric toxins and cause gastroenteritis.[2–6] A healthy adult has to consume ca. 30 g or ml of a food containing 100 to 200 ng toxins produced by $10^{6–7}$ cells/g or /ml; infants and old and sick individuals need lesser amounts. The symptoms occur within 2 to 4 h, with a range of 30 min to 8 h, and are directly related to the potency and amounts of toxin ingested and an individual's resistance. The disease lasts for ca. 1 to 2 d and is rarely fatal. Only four deaths were recorded between 1973 and 1987.[2–7]

The primary symptoms, from stimulation of the autonomic nervous system by the toxins, are salivation, nausea and vomiting, abdominal cramps, and diarrhea. Some secondary symptoms are sweating, chills, headache, and dehydration.[2-7] However, the symptoms and their severity vary among individuals in an outbreak.

E. Food Association

Many foods have been implicated in staphylococcal foodborne outbreaks.[1,2,5,8] In general, the bacterium grows in the food and produces toxins without adversely affecting the acceptance quality. Many protein-rich foods, foods that are handled extensively, foods in which associated bacteria grow poorly, and foods that have been temperature abused are associated with staphylococcal gastroenteritis. Some of the foods that have been more frequently implicated are ham, corned beef, salami, bacon, barbecued meat, salads, baking products containing cream, sauces, and cheeses. Table 24.1 presents the relative frequencies of different foods involved in staphylococcal food poisoning in the U.S. between 1973 and 1987. Pork, baking products, beef, turkey, chicken, and eggs are associated with the high percentages of outbreaks.[1] Different types of salads, because of extensive handling and high chance of temperature abuse, have been implicated in relatively high numbers in staphylococcal food poisoning. From 1983 to 1987, salads were associated with 13% of total staphylococcal food poisoning outbreaks in the U.S. They are included in Table 24.1 in the "other" category. Three major contributing factors in these outbreaks from 1983 to 1987 were improper holding temperature (51.6%), poor personal hygiene (23.4%), and contaminated equipment (17.2%). Major sources of outbreaks (where foods were prepared or served) were food services (24.7%), homes

Table 24.1 Food Types Involved in Confirmed Staphylococcal Food Poisoning Outbreaks from 1973 to 1987 in the U.S.

Food Type	% Outbreaks[a]	Food Type	% Outbreaks
Pork	16.2	Fish	1.3
Bakery products	7.1	Dairy products	1.7
Beef	6.0	Fruits and vegetables	1.1
Turkey	5.5	Ethnic foods	1.1
Chicken	3.8	Other	37.2[b]
Eggs	2.5	Unknown	6.5

[a] Out of a total of 367 outbreaks.
[b] The foods included in the "other" category are not known. However, salads were implicated in many outbreaks. Between 1983 and 1987, salads were involved in ca. 13% of the total staphylococcal foodborne disease outbreaks.

(14.9%), and picnics (8.5%). A high percentage of outbreaks occurred between May and October (63.8%), with the highest frequency in August (21.3%). In the case of imported foods, a raw or processed food exported from a country can have *Sta. aureus* toxins, but can cause food poisoning in a different country. This is exemplified by at least six outbreaks of staphylococcal gastroenteritis in 1989 in the U.S. from the consumption of dishes prepared by using enterotoxin-containing canned mushrooms processed in a plant in the People's Republic of China. *Sta. aureus* probably grew before canning and the enterotoxin, being heat-stable, remained potent after canning.

F. Prevention (Reduction) of the Disease

The normal occurrence of *Sta. aureus* in raw food materials, among food handlers, and many food environments makes it impossible to produce nonsterile foods that are free of this bacterium. Thus, a zero tolerance is not economically possible to achieve. Many foods can contain *Sta. aureus,* and consumption of a food containing 100 or 500 cells/g (or /ml) will, in all probability, not make a person sick (unless the food has large amounts of preformed toxin). To reduce the incidence of staphylococcal food poisoning, the aim is to reduce initial load of *Sta. aureus* in a food by proper selection of the quality of the raw materials and ingredients, sanitation of the food environments, and proper personal hygiene among food handlers.[3–5] People with respiratory diseases, acute types of facial acne, skin rash, and cuts in hands should not handle the food. Where possible, products should be heat-treated to ensure killing of live cells. Following heating, recontamination of the products should be avoided. The most important aim is to chill the processed products and ready-to-eat foods to ≤5°C quickly. Suitable preservatives can also be used to kill or arrest growth. The inside of the food, and not only the surface, should reach the chilled temperature, preferably within 1 h. Finally, the food should not be subjected to temperature abuse and stored for a long period of time at growth temperature before eating. Once heat-stable toxins are formed, heating before eating does not ensure safety.

G. Identification Methods

To associate a food implicated in staphylococcal food poisoning, the food or vomit samples are analyzed for the presence of high levels of enterotoxigenic *Sta. aureus* cells and enterotoxins (see Appendix E). Enumeration technique in one or more selective differential agar media to determine the load of viable cells of *Sta. aureus*, followed by several biochemical tests, such as hemolysis, coagulase, thermonuclease reactions, or ability of a pure culture to produce enterotoxin, are performed to link the potential cause of the food poisoning outbreaks.

Enterotoxins from the food or vomit samples are extracted and tested, either by biological means or by serological means, to associate them with the outbreak. In the biological method, animals (e.g., cats, monkeys, or dogs) are given the enterotoxin preparation orally or it is injected intraperitoneally or intravenously. Vomiting symptoms by the test animals is a positive indication of the presence of staphylococcal enterotoxin.

In the serological methods, the enterotoxins are purified and examined by one of the several recommended immunological methods. Not only are these tests very sensitive, but they allow the identification of the types of enterotoxins involved in a food poisoning case.

H. Analysis of an Outbreak

A foodborne disease outbreak affecting 52 of 101 people who attended a dinner (foods prepared at home) was reported on December 6, 1986, in Riverton, WY.[9] Of these, 49 needed immediate medical attention. The symptoms developed in less than 1 to 7 h after the meal and included nausea (100%), vomiting (98%), diarrhea (90%), abdominal cramps (83%), prostration (62%), chills (52%), sweating (35%), and blood pressure–temperature depression (21%). When the regulatory people came to investigate after 36 h, samples of foods or vomit of the patients were not available from the doctors. However, one vomit sample frozen by a patient was available and, when analyzed, was found to have 12 to 19×10^6/g coagulase-positive *Sta. aureus*. Meat obtained from a leftover turkey carcass had 1×10^6/g coagulase-positive *Sta. aureus*. An investigation revealed that a person who deboned and handled the cooked turkey had an erupting facial rash (acneiform). The turkeys (three total) were improperly cooled following cooking and held on an improperly heated steam table for ca. 4 h before serving.

This is a classical case of staphylococcal food poisoning outbreak, in which more than half the people who ate foods involving several preparations developed many of the classical symptoms, some within 30 min. Although the food samples served in the dinner or vomit samples from the attending physicians or medical facilities were not available, analysis of a vomit sample saved by a patient revealed the presence of very high numbers of coagulase-positive *Sta. aureus* and meat available from a leftover turkey carcass also had fairly large numbers of *Sta. aureus*. Therefore, from the symptoms and indirect evidence, the outbreak was concluded to be an incidence of staphylococcal food poisoning.

The sequences of events were most likely as follows. The erupting facial rash of the food handler was most probably the source of the pathogen. The pathogen was transmitted by the individual through the hands while deboning the cooked turkey. *Sta. aureus* can grow in meat under a suitable environment. The turkey, following cooking and before serving, was temperature abused for a long period of time, thus enabling the contaminants to grow and reach very high populations. However, the acceptance quality of the meat was not adversely affected. The stage was set and many consumed enough toxin with turkey (and possibly with other servings also) to develop the symptoms.[9]

III. BOTULISM

A. Importance

Botulism results following consumption of food containing the potent toxin botulin of *Clostridium botulinum*. It is a neurotoxin and produces neurological symptoms along with some gastric symptoms.[1,2] Unless prompt treatment is administered, it is quite fatal. Infant botulism occurs when an infant ingests *Clo. botulinum* spores, which germinate, grow, and produce toxins in the GI tract and cause specific symptoms.

In the U.S., the average number of outbreaks per year is 15 to 16. From 1973 to 1983, there were 231 outbreaks. This represents ca. 8% (231 out of 2841) of the total reported foodborne outbreaks involving less than 4% (4984 out of 124,994) of the total cases. However, of a total of 247 deaths (2 per 1000 cases) from all foodborne disease, 47 died of botulism during this period (9.5 per 1000 cases) despite the medical treatment available in the country. Most outbreaks occur with foods prepared at home. Ethnic food preparations have been implicated in many cases.

B. Characteristics

1. Organisms

Cells of *Clo. botulinum* strains are Gram-positive rods, occur as single cells or in small chains; many are motile, obligate anaerobes, and form single terminal spores. Cells are sensitive to low pH (<4.6), low A_w (0.93), and moderately high salt (5.5%). Spores do not germinate in the presence of nitrite (250 ppm). Spores are highly heat resistant (killed at 115°C), but cells are killed at moderate heat (pasteurization). Toxins form during growth. Strains can either be proteolytic or nonproteolytic.[9–11]

2. Growth

Clo. botulinum strains, on the basis of the type of toxin production, have been divided into six types: A, B, C, D, E, and F. Of these, A, B, E, and F are associated with human foodborne intoxications. Type A strains are proteolytic, Type E strains are nonproteolytic, but Types B and F strains can be either proteolytic or nonproteolytic.

Proteolytic strains can grow between 10°C and ca. 48°C, with the optimum at 35°C. Nonproteolytic strains grow optimally at 30°C, with a range from 3.3 to 45°C. Optimum growth facilitates optimum toxin production. Anaerobic conditions are necessary for growth. Spore germination and outgrowth before cell multiplication are favored in the environment of cell growth. Similarly, conditions that prevent cell growth also adversely affect spore germination. A pH of 4.6, A_w of 0.93, or 5.5% NaCl can prevent cell growth, but by using two or more parameters along with lower temperature, the lower growth limits of any of the given parameters can be greatly reduced.[9–11]

3. Habitat

Spores of *Clo. botulinum* are widely distributed in soil, sewage, mud, sediments of marshes, lakes and coastal waters, plants, and intestinal contents of animals and fishes. Fruits and vegetables can be contaminated with spores from soil, fishes from water and sediments, and various other foods from many of the given sources. Type A and B spores are more prevalent in soil, sewage, and fecal matters of animals, whereas Type E spores are generally found in marine environments. Type A spores are predominant in western U.S. and Type B spores are found predominantly in eastern U.S. and different parts of the world.[9–11]

C. Toxins and Toxin Production

The toxins of *Clo. botulinum* are neurotoxic proteins. In general, toxins associated with food intoxication in humans (Types A, B, E, and F) are extremely potent, and only a small amount of toxin is required to produce the symptoms and cause death. Following ingestion, toxin molecules are absorbed from the upper portion of the intestine through the intestinal wall and spread via the blood to the peripheral nerves. The toxins block signal transfers, irreversibly causing paralysis of all involuntary muscles. The toxins move slowly through the body. Toxins produced by nonproteolytic strains are not fully activated; trypsin treatment is necessary to activate them (this occurs in the digestive tract). The toxins are heat labile and can be destroyed in a contaminated food by high and uniform heat, such as 90°C for 15 min or boiling for 5 min. Radiation at 5 to 7 mrad can also destroy them.[10,11]

Cell growth is necessary for toxin production. At optimum growth temperature, toxins are produced in large amounts. However, at both extreme growth ranges, enough toxin can be produced by a strain in a food to cause disease and death following ingestion.

D. Disease and Symptoms

Botulism is caused by ingestion of the neurotoxin botulin formed in a food. The toxins are absorbed from the intestine, spread to the peripheral nerves, and block the transmission of impulse. However, at the initial stage (generally 12 to 36 h, but can be 2 h), some gastrointestinal disorders (e.g., nausea, vomiting, diarrhea, and constipation) may be evident. Neurological symptoms develop within a short time, especially if the amount of botulin consumed is high. As they are highly potent

toxins, only a very small amount (1 ng/kg body weight) is necessary for severe symptoms and even death. In general, neurological symptoms include blurred or double vision; difficulty in swallowing, breathing, and speaking; dryness of the mouth; and paralysis of different involuntary muscles, which spreads to the diaphragm, lungs, and heart. Death usually results from respiratory failure.

The toxins are antigenic; thus antitoxins are available. Soon after the onset of the symptoms and if the amount of toxin consumed is fairly low, trivalent antitoxins (against botulin A, B, and E) can be administered to treat the disease. But in some advanced cases, especially if the diagnosis is delayed, antitoxin administration may not be successful. In the U.S., even with available facilities, botulism accounts for ca. 19% of the total foodborne fatal cases.[10–12]

E. Food Association

The events in a foodborne botulism involve contamination of a food with *Clo. botulinum* spores, survival of the spores during processing, and the ability of the spores to germinate and outgrow and the cells to multiply when the product is abused (temperature and time). The largest number of outbreaks are associated with fruits and vegetables (Table 24.2),[1] mainly low-acid vegetables (e.g., green beans, corn, spinach, asparagus, pepper, and mushrooms) and fruits (e.g., figs and peaches). The next highest incidence was with finfish, which includes fermented, improperly cooked, and smoked fish and fish eggs. Type E was associated predominantly with fish and Types A and B were associated with vegetables. The major cause of outbreaks was improper home canning of the contaminated products. Between 1983 and 1987, of the 231 botulism outbreaks in the U.S., 56 occurred at home. The occurrence of botulism from meat, poultry, and dairy products is low, probably because they are mostly heated and eaten quickly. Several outbreaks from unlikely foods (sautéed onions or baked potatoes) have been recorded, but in many instances the foods were subjected to temperature abuse (held for a long time at warm temperature). Some condiments, such as chili peppers, relish, and sauce, have also been associated with outbreaks.

Table 24.2 Food Types Involved in Confirmed Botulism Outbreaks from 1973 to 1987 in the U.S.

Food Types	No.	%	Food Types	No.	%
Beef stew	2	0.9	Mushrooms	5	2.2
Chicken	1	0.4	Beverages (nondairy)	5	2.2
Dairy products	1	0.4	Pork	1	0.4
Finfish	35	15.2	Turkey	1	0.4
Fruits and vegetables	99	42.9	Other[a]	40	17.3
Mexican food	3	1.3	Unknown	38	16.4

[a] Not identified, but probably include several types of food, many of which were of unlikely categories, and no single type was involved in a large number of outbreaks.

Growth of proteolytic strains in meats, and low-acid, high-protein vegetables generally produces obnoxious odors and gas. In low-protein vegetables, these characteristics may not be obvious. Growth of nonproteolytic strains, even in meat, fish, and other high-protein foods, does not produce spoilage characteristics. As the toxins are heat labile, high uniform heating (90°C for 15 min or boiling for 5 min) of a suspected food destroys the toxins and makes the food theoretically safe. But because only a small amount of toxin is enough to cause disease, it is advisable not to consume a suspected food.[1,2,12]

F. Prevention of Botulism

The single most important control method is to use proper temperature and time in home canning of low-acid products.[10–12] Commercial processors use the 13 D concept (Chapter 32). Directions for pressure canning of foods at home are available, and they should be strictly followed. Some foods (e.g., fish) should be properly and uniformly cooked at high temperatures. Foods cooked at temperatures in which spores survive should be stored at low temperatures (at 3°C or below); at refrigerated temperature (4 to 5°C), storage should not be prolonged unless some additional precautions are used, such as NO_2, low pH, low A_w, or NaCl. Suspected foods should be properly heated before consumption, but it is better not to eat them. Even tasting a small amount of a suspected food without giving high and uniform heat treatment can be dangerous.

G. Identification Methods

In a suspected food, the presence of *Clo. botulinum* can be determined by enumeration techniques using selective agar media and anaerobic incubation, followed by biochemical and toxicological testing. The presence of toxins in the food is more often tested. This involves injecting a food extract intraperitoneally into mice. Development of characteristic neurological symptoms, followed by death in 92 h, suggest the presence of toxin. The people engaged in testing for the organism or the toxins need to immunize themselves before handling the materials.

H. Infant Botulism

Clo. botulinum spores, ingested by human infants through food and the environment, can germinate in the intestine and produce the toxin to cause infant botulism.[13] The spores fail to produce the same disease in individuals above 1 year of age, probably because the well-established normal population of gastrointestinal flora discourages spore germination and cell multiplication by *Clo. botulinum*. Both Types A and B have been identified in infant botulism cases. Symptoms include general weakness, inability to suck and control the head, loss of reflexes, and constipation. Foods such as honey and corn syrup and dirt have been linked as sources of *Clo. botulinum* spores in infant botulism cases.[13]

I. Analysis of a Foodborne Botulism Case

On May 4, 1992, in New Jersey, a man of Egyptian origin developed dizziness, facial drooping, dry mouth, weakness, and respiratory difficulties. Within 2 days, two more family members developed similar symptoms. The cases were diagnosed as botulism and treated with trivalent (A, B, E) botulinal antitoxins. The source of the toxin was traced to an ethnic fish preparation made with uneviscerated, salt-cured fish. The previous day, the family had obtained the product from outside and consumed it without cooking.

The sequence of events was most probably as follows. The fish had *Clo. botulinum* spores (probably Type E) in the gut from the water and water sediment. The uneviscerated fish were cured in salt, the concentration of which was not high enough to prevent germination, cell growth, and toxin production. Without cooking, the product was consumed, probably in different amounts, by the family members, who developed symptoms at different times. The outbreak could have been avoided by removing the viscera, curing the fish in recommended salt concentrations, and heating before eating.[14]

IV. MYCOTOXICOSIS

A. Importance

Many strains of molds, while growing in a suitable environment (including in foods), produce metabolites that are toxic to humans, animals, and birds, and are grouped as mycotoxins.[15,16] Consumption of foods containing mycotoxins causes mycotoxicosis. They are secondary metabolites and not proteins or enteric toxins. Many are carcinogens and, when consumed, can cause cancer in different tissues in the body. Some cause toxicity of organs by unknown mechanisms. Incidents of mycotoxicosis in humans have not been recorded in recent years in many countries, at least in the developed ones. This is because their presence in many foods is critically regulated and evaluated. Incidents of mycotoxicosis have been recorded in some developing countries in recent years. Some well-known mycotoxicosis incidents in humans in the past are ergotism from consumption of bread made from rye infected with *Claviceps purpurea* in Europe between the 14th and 16th centuries, yellow rice disease from the consumption of rice infested with toxigenic strains of several *Penicillium* species in Japan during the 17th century, and alimentary toxic aleukia from the ingestion of grains infested with toxigenic strains of *Fusarium* species in Russia in the early 20th century. In recent years, several incidents were reported in animals and birds, which include the death of thousands of turkeys from liver necrosis in the 1960s in England following feeding peanut meal in which *Aspergillus flavus* grew and produced the toxin aflatoxin.

B. Characteristics

1. Organisms

Toxigenic species and strains of molds from many genera are known to produce mycotoxins. Toxigenic strains from several species and genera and the toxins they produce include *Asp. flavus, Asp. parasiticus* (both produce aflatoxins), *Asp. nidulans*, *Asp. virsicolor* (sterigmatocystin), *Penicillium viridicatum* (ochratoxin), *Pen. patulum* (patulin), *Pen. roquefortii* (roquefortin), and *Cla. purpurea* (ergotoxin). Toxigenic strains cannot be differentiated from nontoxigenic strains by morphological characteristics only. It is necessary to grow a strain under suitable conditions and test the material for the presence or absence of a mycotoxin. This is particularly important for the mold strains from different genera that are used in food production.[15]

2. Growth

In general, molds grow best in humid and warm environments. They are aerobic and thus need air for growth. They can grow, though slowly, at very low A_w (0.65), low temperature (refrigerated temperature), and low pH (pH 3.5). These conditions are often used to extend the shelf life of many foods. Unless other methods (such as vacuum packaging) are used, they can grow in these foods, and, if toxigenic, can produce toxins in the foods.[15]

3. Habitat

The spores are present in soil, dust, and the environment. Many foods can have viable spores or mycelia, especially before a heat treatment.[15]

C. Toxins and Toxin Production

Mycotoxins include a large number of toxins produced by different toxigenic species and strains of molds.[15,16] Many have not yet been identified. Some of the toxins have been listed before. They are small-molecular-weight heterocyclic organic compounds and some have more than one chemical type. An example is aflatoxin, which has two major types, B1 and G1, and each has several subtypes. Aflatoxin B1 is considered the most potent.

Mycotoxins are produced by toxigenic mold strains as secondary metabolites. Toxin production, in general, is directly related with the growth rate of a mold strain. In microbiological media suitable for growth of molds, *Asp. flavus* strains can produce optimum concentrations of aflatoxin at 33°C, pH 5.0, and A_w 0.99.[15,16]

D. Food Association

The growth of toxigenic mold strains and the presence of specific mycotoxins have been detected in many foods, such as corn, wheat, barley, rye, rice, beans, peas,

peanuts, bread, cheeses, dry sausages, spices, apple cider, grain meals, dough cassava, cotton seeds, and spaghetti.[15,16] Consumption of mycotoxin-contaminated food can cause mycotoxicosis in humans. Feeding moldy products to food animals (including moldy silage) and birds can also produce foods of animal origin (milk, eggs) that are contaminated with mycotoxins. Many mycotoxins are resistant to heat used in the normal preparation of foods. Thus, their elimination by heating is not used to remove them from foods. In the U.S., regulatory agencies have set limits of aflatoxins in peanut butter (<20 ppb) and milk (<2 ppb).

E. Prevention of Mycotoxicosis

To prevent human mycotoxicosis, the contamination of food with toxigenic mold strains (or all molds, unless necessary) should be reduced.[15,16] This is relatively difficult to achieve, but proper packaging can be used to reduce the incidence. Heat treatment, where possible, can also reduce the load by killing the molds and their spores. Preventing growth in food (and feeds) should be a major consideration in reducing the incidence of human mycotoxicosis. This can be achieved by using anaerobic packaging; reducing A_w, where possible, to 0.6 or below; freezing; and using specific preservatives against mold growth. A product in which molds have grown should not be consumed. Although trimming of foods showing mold growth is a common practice, one cannot be sure whether the remaining portion is free of mycotoxins. Under commercial operations, removal of some mycotoxins (aflatoxins) from food or food ingredients has been studied. Generally, solvent extraction methods have been used. Finally, treatment of some foods or food ingredients with suitable chemicals to inactivate mycotoxins, such as aflatoxins, has also been studied. Some chemicals (e.g., ammonia, hydrogen peroxide, and sodium hypochlorite) were found to inactivate aflatoxins.

F. Detection Methods

Several methods have been studied to detect mycotoxins, more particularly, aflatoxins in food. These include solvent extraction of a suspected food sample, thin layer chromatography of the extract, and visualizing under a UV light or a fluorescent light. Chemical tests and analysis by mass spectral methods are used to identify the specific type of aflatoxins.[15,16]

V. CONCLUSION

Foodborne pathogenic microorganisms producing toxins while growing in food and food ingredients are involved in human foodborne intoxication. The toxins can be proteins or small organic molecules that are heat labile or heat stable. A toxin, depending on the pathogen, can produce only enteric symptoms or neurological symptoms or cancer. The incidence of foodborne intoxication can be reduced by using proper sanitation and preserving foods during processing and subsequent handling.

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QUESTIONS

1. List five characteristics of foodborne intoxication.
2. Describe some growth characteristics of *Sta. aureus* strains that give them an advantage for growth and toxin production in a food. Name two known food types that have the highest incidence of staphylococcal poisoning and state the predisposing causes.
3. List the characteristics of the enterotoxins of *Sta. aureus* and discuss the mechanisms by which they produce enteric symptoms.
4. List the symptoms of staphylococcal food poisoning, and discuss how the disease can be differentiated from other food poisoning of microbial origin.
5. From a case of staphylococcal food poisoning outbreak (get it from the instructor or the Internet), determine the sequence of events in the outbreak. What precautions could have been taken to avoid the incidence?
6. Describe how foodborne botulism differs from infant botulism.

7. List the types of *Clo. botulinum* associated with food intoxication, and describe the changes that can occur from their growth in a food high in protein and a food low in protein.
8. List the toxins associated with foodborne botulism in humans and describe the mechanisms by which the toxins produce neurological symptoms.
9. Discuss how a combination of several environmental parameters can be used to control growth of *Clo. botulinum* in a food.
10. List two food groups frequently associated with botulism in the U.S. Suggest four methods to reduce the incidence.
11. Explain the terms mycotoxins, mycotoxin-producing strain of *Penicillium* species, aflatoxin, and growth characteristics of molds.
12. List five foods that directly or indirectly can be contaminated with mycotoxins. Discuss mycotoxin concerns in foods and food ingredients imported to the U.S. from other countries.

CHAPTER 25

Foodborne Infections

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I. INTRODUCTION

Foodborne infection occurs from the consumption of food (and water) contaminated with pathogenic enteric bacteria and viruses. Many pathogens are included in this group. However, many are involved more frequently than others, and they are discussed in this chapter. The discussions include their relative importance, characteristics, food association, toxins, disease symptoms, and prevention. For some, detection methods and case histories are also included.

The following are some characteristics of foodborne infections:

1. Live cells of the enteric pathogens (bacteria and viruses) have to be consumed through food.
2. The surviving cells (from gastric environment) penetrate through the membrane and establish in the epithelial cells of the intestines, multiply, and produce toxins (infection).
3. Dose levels that cause infection vary greatly. Theoretically, one live cell has the potential to produce the disease. Experts estimate that consumption of ca. 10 cells (for an extremely virulent species and strain, such as *Escherichia coli* O157:H7) to ca. 10^5 cells or more (for a less virulent species and strain, such as *Yersinia enterocolitica*) might be required for the disease.
4. Symptoms generally occur after 24 h, which, depending on the pathogen, and can be both enteric and nonenteric in nature.
5. Enteric symptoms are local and due to enteric infection and the effect of toxins. Symptoms include abdominal pain, diarrhea (sometimes accompanied with blood), nausea, vomiting, and fever. Examples of pathogens include *Salmonella*, *Shigella*, enteroinvasive *Esc. coli* (EIEC), *Vibrio parahaemolyticus, Campylobacter jejuni*, and *Yer. enterocolitica*.
6. Nonenteric symptoms (along with enteric symptoms) result when the pathogens or their toxins pass through the intestine and invade or affect other internal organs

and tissues. Symptoms depend on the types of organs and tissue affected, but are accompanied by fever. Examples of pathogens include *Listeria monocytogenes*, enterohemorrhagic *Esc. coli* (EHEC), *Vib. vulnificus*, and Hepatitis A virus.

II. SALMONELLOSIS BY *SALMONELLA*

A. Importance

Before the 1940s, *Salmonella typhi* and *Sal. paratyphi* were considered the major causes of worldwide foodborne and waterborne diseases caused by *Salmonella* in humans. However, with the pasteurization of milk and chlorination of water supplies, the spread of typhoid and paratyphoid fever through food and water was greatly reduced, at least in the developed countries. As efficient techniques to isolate and identify other *Salmonella* serovars from foods and environmental samples were developed, it became apparent that the worldwide incidence of foodborne salmonellosis caused by other *Salmonella* is quite high. Since the 1950s, foodborne salmonellosis has been recognized to be the major cause of all foodborne diseases by pathogenic bacteria and viruses, both in number of incidents (sporadic and outbreaks) and number of cases. Although, at present, scientific information about the habitats, mode of transmission in foods, growth characteristics, and survival parameters of the pathogen are available, and methods to control its contamination of foods have been developed, foodborne salmonellosis is still the leading cause of foodborne bacterial and viral diseases in the U.S. and other developed countries. This is quite puzzling. The control measures seem to be working for several other foodborne pathogens, at least in the U.S., such as *Clostridium perfringens, Bacillus cereus, Yer. enterocolitica*, *Vib. parahaemolyticus*, and probably *Staphylococcus aureus*. Even incidences from *Sta. aureus* and *Lis. monocytogenes* seemed to have declined since the 1990s. It is somewhat astonishing that control measures are not working against *Salmonella*. Between 1969 and 1976, the average number of reported foodborne salmonellosis outbreaks was ca. 37 per year in the U.S. In contrast, between 1983 and 1987, the average number of outbreaks per year was more than 68. Not only does the incidence not decrease, but it continues to increase at a high rate. It is difficult to pinpoint the exact causes of this increase. It may be related to the large number of serotypes present in high frequency in carrier state in food animals, birds, pets, insects, humans, and their ability to grow in foods; the way the food animals and birds are raised, processed, and marketed; better surveillance systems by regulatory agencies; or our lifestyles and food habits giving these pathogens an edge.[1–3]

Tauxe[3] indicated that the present increase in salmonellosis (including foodborne salmonellosis) in the U.S. could be related to four factors: (1) increase in number of antimicrobial-resistant *Salmonella* isolates, (2) increase in immunodeficient individuals who are extremely susceptible to *Salmonella*, (3) increase in egg-associated *Salmonella* Enteritidis contamination due to increase in laying hens with infected ovaries, and (4) food production in centralized facilities that can lead to, if contamination occurs, extremely large and widespread outbreaks. It is necessary to under-

stand the importance of these factors in the increase of salmonellosis and to develop corrective measures to control the incidence.

There are more than 2000 serovars (based on somatic, flagellar, and capsular antigen types) of *Salmonella*, potentially capable of causing salmonellosis in humans. Along with fecal–oral direct transmission, contaminated food and water can cause salmonellosis. Recently, a better system of nomenclature has been developed to group these serovars in a few species instead of considering each as a separate species.

1. Current Nomenclature System

The serovars of *Salmonella* have a high degree of relatedness in their DNAs. It was proposed on this basis that the genus *Salmonella* should have only one species, *Salmonella enterica*, and six subspecies, two of which are from the *Arizona* group (Table 25.1). The specific epithets that were used before (e.g., *typhimurium* for *Sal. typhimurium*) should be considered as the serotypic name, and the strains of a serotype can be distinguished by numerals, letters, and a combination, as previously used. It is recommended that in formal publications, the complete name of the species and subspecies be given (e.g., *Salmonella enterica* subsp. *enterica*). Then serotypic names can be used (e.g., ser Typhimurium; not italicized). In informal communications, cultures may be reported in familiar terms (e.g., *Salmonella* Typhimurium).[4]

In this chapter, the previous method has been replaced for most serovars (e.g., *Salmonella* Typhimurium). The two serotypes associated with frequent incidents of foodborne illness of *Salmonella*, *Sal.* Typhimurium, and *Sal.* Enteritidis, are in Subspecies 1.

2. Predominant Serotypes in Salmonellosis

Among the six subspecies *Salmonella enterica*, subsp. *enterica* includes most serotypes frequently associated with foodborne salmonellosis. However, two of the more than 2000 serotypes, serotype Typhimurium and serotype Enteritidis, are involved in higher frequencies worldwide. Many animals, including food animals and pets,

Table 25.1 Six Subspecies of Genus *Salmonella*

1.	*Salmonella enterica* subsp.[a] *enterica*
2.	*Salmonella enterica* subsp. *salamae*
3a.	*Salmonella enterica* subsp. *arizonae*
3b.	*Salmonella enterica* subsp. *diarizonae*
4.	*Salmonella enterica* subsp. *houtenae*
5.	*Salmonella enterica* subsp. *bongori*

[a]The relationship between this subspecies scheme and Kauffman's subgenus scheme are as follows: Subspecies 1 corresponds to Subgenus I, Subspecies 2 corresponds to Subgenus II, Subspecies 3a and 3b correspond to Subgenus III and *Arizona hinshawii,* Subspecies 4 corresponds to Subgenus IV, and Subspecies 5 constitutes six atypical serotypes from Subgenus II and IV.

harbor serotype Typhimurium in a carrier state, whereas serotype Enteritidis can infect the ovaries of poultry, especially chicken, and can be transmitted through eggs.

Because of the increase in antibiotic use in feed of food animals and birds, there is an increase in multidrug-resistant (MDR) strains among *Sal. enterica* serotypes. One that has created concern is the MDR *Salmonella* Typhimurium definitive phage type (DT) 104. The strains are resistant to several antibiotics, including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. It is suspected that they are transferred among strains or serovars by different methods of genetic recombinations, especially under antibiotic selective pressure.[5]

During the last 10 years, foodborne salmonellosis from *Salmonella* Enteritidis has increased greatly, and, at present, the frequency of incidence from it is as high as that caused by *Salmonella* Typhimurium. Among the strains, serotype Enteritidis phage type (DT) 4 and several other types are involved in salmonellosis in high frequency. One of the reasons for this could be the way the poultry is raised.

B. Characteristics

Salmonella cells are Gram-negative, nonsporulating, facultative anaerobic, motile rods. They form gas while growing in media containing glucose. Generally, they ferment dulcitol, but not lactose; utilize citrate as a carbon source; produce hydrogen sulfide, decarboxylate lysine, and ornithine; do not produce indole; and are negative for urease. They are mesophilic, with optimum growth temperature between 35 and 37°C, but generally have a growth range of 5 to 46°C. They are killed by pasteurization temperature and time, sensitive to low pH (4.5 or below), and do not multiply at an A_w of 0.94, especially in combination with a pH of 5.5 and below. The cells survive in frozen and dried states for a long time. They can multiply in many foods without affecting the acceptance qualities.[6,7]

C. Habitat

Salmonellae are natural inhabitants of the gastrointestinal tracts of domesticated and wild animals, birds, and pets (including turtles and frogs), and insects. In animals and birds, they can cause salmonellosis and then persist in a carrier state. Humans can also be carriers following an infection and shed the pathogens through feces for a long time. They have also been isolated from soil, water, and sewage contaminated with fecal matters.[6,7]

D. Toxins

Following ingestion of *Salmonella* cells, the pathogens invade mucosa of the small intestine, proliferate in the epithelial cells, and produce a toxin, resulting in an inflammatory reaction and fluid accumulation in the intestine. The ability of the pathogens to invade and damage the cells is attributed to the production of a thermostable cytotoxic factor. Once inside the epithelial cells, the pathogens multiply and produce a thermolabile enterotoxin that is directly related to the secretion of

fluid and electrolytes. Production of the enterotoxin is directly related to the growth rate of the pathogens.[6,7]

E. Disease and Symptoms

Human salmonellosis is different from typhoid and paratyphoid fever caused by *Sal. typhi* and *Sal. paratyphi*. Although some *Salmonella* serovars are specific against different animals and birds, all are considered to be potential human pathogens capable of causing salmonellosis. For foodborne salmonellosis, an individual generally has to consume ca. 10^{5-6} cells; however, for some virulent strains, ingestion of fewer cells can cause the disease. Strains sensitive to gastric acidity generally need more cells to establish in the intestine and cause the disease; conversely, acid-resistant strains may require fewer cells to cause the disease. Following ingestion of the pathogen, symptoms appear in 8 to 42 h, generally in 24 to 36 h. Symptoms last for 2 to 3 days, but in certain individuals can linger for a long time. An individual remains in a carrier state for several months following recovery.

Not all individuals ingesting the same contaminated foods develop symptoms, and those who develop symptoms do not have all the symptoms in the same intensity. Susceptibility varies with the state of health and natural resistance of an individual. The general symptoms are abdominal cramps, diarrhea, nausea, vomiting, chills, fever, and prostration. It can be fatal, especially to the sick, infants, and the elderly.[6,7]

F. Food Association

Foods of animal origin have been associated with large numbers of outbreaks. These include beef, chicken, turkey, pork, eggs, milk, and products made from them. In addition, many different types of foods have been implicated in both sporadic cases and outbreaks (Table 25.2). These foods were contaminated directly or indirectly with fecal matters from carriers (animals, birds, and humans) and eaten either raw or improperly cooked, or contaminated following adequate heat treatment. Cross-contamination at home and at food services are the major sites of contamination of heated foods with *Salmonella*. Salmonellae have also been isolated from many foods of plant origin (use of sewage as fertilizer or washing products with polluted water), seafood, and finfish (harvested from polluted water).[1,2]

Table 25.2 Foods Associated with Salmonellosis Outbreaks from 1973 to 1987 in the U.S.

Food	Number of Outbreaks[a]	Food	Number of Outbreaks
Beef	77	Bakery products	12
Chicken	30	Fruits and vegetables	9
Turkey	36	Beverages	4
Pork	25	Chinese food	2
Eggs	16	Mexican food	10
Dairy products	50	Other foods	191
Shellfish and finfish	8	Unknown	320

[a] Total number of outbreaks, 790; and number of cases, 55,864; number of deaths, 88.

Although there are more than 2000 serotypes of *Salmonella*, only some of them have been frequently associated with foodborne illnesses. This can be because of geographical distribution of the serotypes as well as pathogenicity of a serovar or a strain. In the U.S., *Salmonella* Typhimurium has been considered the major causative agent of foodborne salmonellosis (more than 20% of the total cases). However, since the 1980s, foodborne salmonellosis from *Salmonella* Enteritidis has increased, mainly from contaminated Grade A shell eggs. In recent years, it has been involved in the same number of cases as *Salmonella* Typhimurium. The exact cause of the predominance of *Salmonella* Enteritidis is not yet clearly understood.[6,7] The methods used in raising food of animals and birds and in processing of foods of animal origin are suspected to have an important role.

G. Prevention and Control

Raw foods of animal origin that are heat treated before consumption can have *Salmonella*. However, in the U.S. (and other developed countries), as per regulatory requirements, heat-treated and ready-to-eat foods that contain *Salmonella* in portions (samples) tested are considered to be adulterated and should not be sold. Many food-processing industries have in-house *Salmonella* (as well as several other pathogens) surveillance programs to control the presence of *Salmonella* in their products. Regulatory agencies also have programs to educate consumers at home and food handlers in food service places to control *Salmonella* contamination in foods. These include proper cooking of foods (minimum to pasteurization temperature and time, such as 71.7°C for 15 sec or equivalent) and prompt cooling (to 3 to 4°C or freezing, if not used in 2 h); preventing cross-contamination of ready-to-eat food with a raw food through cutting boards, equipment, utensils, and hands; using proper sanitation and personal hygiene; sick people not handling a food; and properly reheating a food refrigerated for a long time.[6,7]

H. Detection Method

The methods involve preenrichment of a sample of food in a nutrient broth, followed by selective enrichment, streaking on a selective-differential agar medium, and biochemical and serological confirmation (see Appendix E). Several rapid methods, based on specific immunological characteristics and nucleotide base sequence in nucleic acids, have been developed.

I. Case Study

In July 1989, 21 out of 24 people who attended a baby shower at a home in New York had gastroenteritis, with severe diarrhea, vomiting, fever, and cramps 6 to 57 h after the party.[8] Twenty people needed medical help, of which 18 were hospitalized; one at 38 weeks of pregnancy delivered while ill and the infant developed septicemia. *Salmonella* Enteritidis was isolated from all 21 people (rectal swab) and the infant. All 21 ill attendees, but not the 3 who remained well, ate a homemade baked ziti pasta dish consisting of one raw egg, ricotta cheese, cooked tomato, and meat sauce,

mixed together in a pan and refrigerated overnight. Before serving, the preparation was baked for 30 min at 350°F (176.7°C). Several attendees commented that the center of the ziti was cold when served. *Salmonella* Enteritidis was isolated from the leftover baked ziti and from the unused eggs from the carton. *Salmonella* Enteritidis was also isolated from laying hens at the poultry farm that supplied the eggs.

The poultry farm should have tested the birds and culled those carrying the pathogen as a regulatory requirement. At home, proper methods should have been used to prepare the dish to avoid the incident. Because only one egg was used for a serving of 24+ people, it is likely that *Salmonella* Enteritidis was present initially in the food in small numbers and multiplied before or during (slow) refrigeration. Also, if the dish was taken out of the refrigerator a long time before baking, *Salmonella* cells could have multiplied. During the 30-min baking, the content was not thoroughly and evenly heated (the center was cold), so some *Salmonella* Enteritidis cells survived, and when ingested caused salmonellosis. The dish could have been prepared then and baked immediately after mixing for a longer time for the food to attain a high and uniform temperature and then refrigerated quickly. It could have been reheated to a high and uniform temperature before serving to prevent the salmonellosis outbreak.

III. LISTERIOSIS BY *LISTERIA MONOCYTOGENES*

A. Importance

Human listeriosis has been recognized for a long time. However, the presence of *Lis. monocytogenes* in many foods of animal and plant origin and illnesses resulting from consumption of contaminated foods was recognized rather recently.[9–13] Human listeriosis is considered by some to be an opportunistic disease. Individuals with normal health may not develop symptoms or show a very mild enteric form of the disease. However, it is highly fatal (30 to 40%) to fetuses, newborns, infants, the elderly, pregnant women, and immunocompromised people, such as those with cancer, renal diseases, heart diseases, and AIDS. In addition, its ability to grow in many foods at refrigerated temperature helps the organism reach from a low initial level to an infective dose level during storage of refrigerated foods, which include those that originally harbored the pathogen and those that were postheat contaminated. The increase in consumption of many types of ready-to-eat foods stored for fairly long periods of time, and consumption of foods without properly reheating or by microwave heating, has increased the likelihood for the pathogen to cause the disease. Many technological developments used for the production of ready-to-eat foods may have steps that can contaminate food with the pathogens in low levels, which then can reach a higher level during subsequent refrigerated storage before consumption. Any temperature abuse, even for a short time, can accelerate the growth rate.

It is clear that many of the conditions given have given an advantage to *Lis. monocytogenes* to become a newly emerging foodborne pathogen in many countries.

However, an understanding of the type of foods that are mostly involved in listeriosis, the food processing steps that can contaminate ready-to-eat foods with this pathogen, and the special groups of people most susceptible to the disease helped regulatory agencies develop procedures to reduce the number of foodborne listeriosis. These have been achieved through changes in the processing steps and testing of foods so that contaminated ready-to-eat foods do not reach consumers, and by educating susceptible consumer groups about food choices, eating habits, and sanitary practices in food preparation. As a result, in developed countries, the number of listeriosis cases has dropped; in the U.S., the number of human listeriosis cases has fallen from 2000 cases in 1986 to ca. 1000 cases in 1991.

The genus *Listeria* contains several species, of which *Lis. monocytogenes* is considered to be pathogenic. The species has several serogroups: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c and 4b. Whereas 1/2a and 1/2b were the predominant serogroups isolated in foodborne human listeriosis in Europe, 4b was predominant in Canada and the U.S.

B. Characteristics

Lis. monocytogenes is a Gram-positive, psychrotrophic, facultative anaerobic, non-sporulating, motile, small rod. In fresh culture, the cells may form short chains. It is hemolytic and ferments rhamnose but not xylose.

Lis. monocytogenes is a psychrotroph and grows between 1 and 44°C, with optimum growth at 35 to 37°C. At 7 to 10°C, it multiplies relatively rapidly. It ferments glucose without producing gas. It can grow in many foods and environments. The cells are relatively resistant to freezing, drying, high salt, and pH 5.0 and above. They are sensitive to pasteurization temperature (71.7°C for 15 s or 62.8°C for 30 min), but when inside the white blood cells, a temperature of 76.4 to 77.8°C for 15 s is required to kill the cells.[9,10,12]

C. Habitat

Lis. monocytogenes is isolated from many environmental samples, such as soil, sewage, water, and dead vegetation. It is isolated from the intestinal contents of domesticated animals and birds. Humans can also carry the organisms in the intestine without any symptoms. A large proportion of uncooked meat, milk, egg, seafoods, and fish, as well as leafy vegetables and tubers (potatoes and radishes, in particular), contains *Lis. monocytogenes*. Many heat-processed foods, such as pasteurized milk and dairy products, and ready-to-eat meat preparations also contain the organism. *Lis. monocytogenes* is isolated in high frequency from different places of food processing and storage areas.[10,14]

D. Toxin

The virulence factor of *Lis. monocytogenes* is a specific type of hemolysin, listeriolysin O. It is produced during the exponential growth of the cells. The pathogens invade different body tissues and multiply inside the body cells, releasing the toxin. The toxin causes death of the cells.[9,10,14]

E. Disease and Symptoms

People with normal health, following ingestion of a food contaminated with *Lis. monocytogenes*, may or may not produce symptoms. Most often, symptoms appear 1 to 7 d following ingestion and include mild flu-like symptoms with slight fever, abdominal cramps, and diarrhea. The symptoms subside in a few days, but the individual sheds *Lis. monocytogenes* in the feces for some time.[9,10,14]

The symptoms among sensitive groups are quite different. These groups include pregnant women, unborn fetuses, infants, elderly people with reduced immunity due to diseases, and people taking special medications, such as steroids and chemotherapeutic agents. Initial symptoms are enteric, with nausea, vomiting, abdominal cramps, and diarrhea, along with fever and headache. The pathogens then spread through the bloodstream and invade tissues in different vital organs, including the central nervous system. In pregnant women, the pathogen can invade organ tissues of the fetus through the placenta. Symptoms include bacteremia (septicemia), meningitis, encephalitis, and endocarditis. The fatality rate in fetuses, infected newborn infants, and immunocompromised individuals is very high. The infective dose is considered to be ca. 100 to 1000 cells, particularly for the highly sensitive individuals.

F. Food Association

Foodborne listeriosis in humans is mainly sporadic; however, outbreaks have been reported from the consumption of contaminated cole slaw, pasteurized milk, raw milk and dairy products, soft cheeses (Mexican-style, Brie, and Liederkranz), meat pâté, turkey franks, cold cut meats, improperly cooked chicken, and smoked mussels. Sporadic listeriosis was also documented from the consumption of these foods either at home or at delicatessen counters. Heat-treated foods were either not properly heated or were contaminated following heating. As many raw foods of both animal and plant origin harbor *Lis. monocytogenes*, consumption of raw foods or recontaminated heat-processed foods has caused listeriosis. Growth during long refrigerated storage and temperature abuse before eating have been implicated in many cases. Individuals suffering most from listeriosis and fatality from the disease were from the sensitive groups.[9,10,14]

G. Prevention and Control

Because of the ubiquitous presence of *Lis. monocytogenes*, it is impossible to have foods free of this pathogen. However, in many countries, a strong *Listeria* control program at the commercial production facilities has been imposed by regulatory agencies and industries. This has helped greatly reduce the number of listeriosis cases since 1991. However, in recent years, there has been an increase in incidents of sporadic and outbreaks of listeriosis as well as in product recalls. In 1999, there were 24 recalls. In the U.S., if the presence of *Lis. monocytogenes* is found in 25 g-portions of cooked, ready-to-eat meat and poultry products, each lot is considered adulterated and discarded. A plant producing contaminated products is then thoroughly tested

and subjected to proper sanitation (hazard analysis critical control point; see Appendix D) until products free of *Lis. monocytogenes* are produced. Because of the effectiveness of current regulatory procedures, it is possible that in the future the current stringent zero-tolerance may be slightly relaxed.[10–14]

In addition to the control measures in processing facilities, regulatory agencies have stressed on consumer education to reduce foodborne listeriosis. This includes thoroughly cooking raw foods of animal origin; thoroughly washing raw vegetables before eating; keeping uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods; not consuming raw milk or foods made with raw milk; and washing hands, knives, and cutting boards after handling uncooked foods. In addition, there are special recommendations for high-risk individuals: avoid soft cheeses (Mexican style, Feta, Brie, Camembert, blue-veined, cream or cottage cheeses); reheat (until steaming) all refrigerated leftover foods and ready-to-eat foods before eating; avoid foods from the delicatessen (recommended for pregnant women, elderly people, and immunocompromised people).[10–14]

H. Detection Method

The most commonly used method involves preenrichment and enrichment steps in recommended broths and streaking on specific selective-differential agar media plates. Suspected colonies are then tested for biochemical and serological profiles. Several rapid methods have also been developed based on immunological characteristics and nucleic acid base sequences (see Appendix E).

I. Case Study

In December 1988 in Oklahoma, a cancer patient developed septic listeriosis after consumption of turkey franks following heating in a microwave oven.[15] *Lis. monocytogenes* serotype 1/2a was isolated from the patient, from the remaining franks in the opened package (in high numbers) in the refrigerator, and several other foods in the same refrigerator. Isolation of the same serotype from unopened packages (in low numbers) of the same brand of turkey franks led the regulatory agency to believe that the contaminated turkey franks were the cause of this foodborne listeriosis. Subsequent investigations by the regulatory agencies after 4 months revealed that the plant processing this brand of franks had the same serotype of *Lis. monocytogenes* (as well as other serotypes) in the processing environment, and the heated franks were recontaminated from the conveyor belt following peeling of casings and before repackaging (see Chapter 28).

This incident reveals several important aspects of foodborne human listeriosis: (1) the person affected with listeriosis is immunocompromised; (2) ready-to-eat franks were the source of the pathogenic serotype; (3) microwave heating, the way used by the individual, did not kill the pathogen; (4) the pathogen grew during refrigerated storage in the franks; (5) several other foods in the same refrigerator were also cross-contaminated; (6) the pathogenic strain was present in the processing environment (along with other serotype) even after 4 months; and (7) recontamination of franks following heating occurred from contaminated equipment before packaging.

To control this incident of listeriosis, proper sanitation of the processing plant would be the primary requirement. At the consumer level, the incident indicates the importance of proper heating of the product before eating (and prevention of cross-contamination of refrigerated ready-to-eat foods, especially by the high-risk individual).

IV. PATHOGENIC *ESCHERICHIA COLI*

A. Importance

Since its discovery in 1885, *Escherichia coli* was considered a harmless, Gram-negative, motile, nonsporulating, rod-shaped, facultative anaerobic bacterium, a normal inhabitant of the intestinal tract of humans and warm-blooded animals and birds. Because it is normally present at a very high level (in millions per gram of the content of the large intestine), for a long time it has been used as an index organism for possible fecal contamination and presence of enteric pathogens in food and water (see Chapter 29). Since the mid-1940s, evidence has accumulated that certain *Esc. coli* strains cause diarrhea, particularly in infants, and they were designated as enteropathogenic *Esc. coli*. However, current evidence indicates that pathogenic strains of *Esc. coli* are more than one type. They are subdivided into four groups.[16,17] Recently, they have been subdivided into six groups, including the four discussed here, together with the enteroaggregative *Esc. coli* ($EA_{gg}EC$) and diffuse-adhering *Esc. coli* (DAEC).

1. *Enteropathogenic* Esc. coli *(EPEC)*

These strains are important in infant diarrhea worldwide, especially in places with poor sanitation. They are transmitted directly or indirectly through human carriers. Several serotypes are implicated in waterborne and foodborne disease outbreaks in different countries. The pathogenesis is produced by their ability to form a close physical contact with the intestinal epithelial cells and cause lesions. One needs to ingest high numbers of cells (10^{6-9}) to develop symptoms. The predominant symptom is gastroenteritis.

2. *Enterotoxigenic* Esc. coli *(ETEC)*

These strains are the major cause of diarrhea in travelers, as well as in infants in many developing countries with poor sanitation. The pathogens produce an invasive factor and a heat-labile (HL), a heat-stable (HS), or both HL and HS, enterotoxins to cause the disease. The symptom is gastroenteritis, like a mild form of cholera. The pathogen is spread directly or indirectly by human carriers. Both food and water have been implicated in outbreaks and sporadic cases in humans. In 1983, imported Brie cheese contaminated with O27:H7 serotype caused outbreaks in several countries, including the U.S. Ingestion of large numbers of cells (10^{8-9}) is necessary for an individual to develop symptoms.

3. *Enteroinvasive* Esc. coli *(EIEC)*

These strains are known to cause dysentery similar to that that caused in shigellosis. The ability of the strains to produce an invasive factor is thought to be the cause of the disease. Human carriers, directly or indirectly, spread the disease. Ingestion of as many as 10^6 cells may be necessary for an individual to develop symptoms. An outbreak in the U.S. as early as 1971 was recognized from the consumption of imported Camembert cheese contaminated with serotype O124:B17.

4. *Enterohemorrhagic* Esc. coli *(EHEC)*

The strains in this group (a principal serogroup is O157:H7) have been recognized relatively recently as the cause of severe bloody diarrhea (hemorrhagic colitis) and hemorrhagic uremic syndrome (HUS) in humans. Animals, particularly dairy cattle, are thought to be the carriers. Ingestion of as few as 10 to 100 cells can produce the disease, especially in sensitive individuals. Three enterotoxins (verotoxins) produced by the serotype cause the disease symptoms.

Although there is no clear differentiation for these four subgroups, both EIEC and EHEC strains seem to fit with the enteric pathogens associated with foodborne infection. Thus, these two groups are discussed in this chapter. Both EPEC and ETEC groups are included in Chapter 26, under toxicoinfection.

B. Gastroenteritis Due to EIEC

1. Toxins

The pathogens produce several polypeptides, the genes of which are encoded in a plasmid. These are considered to be the invasive factors that enable the pathogen to invade epithelial cells and set up infection in the colon. Separate toxins have not yet been identified.[16,17]

2. Disease and Symptoms

The disease and symptoms are similar to those for shigellosis. Following ingestion of the pathogen (ca. 10^6 cells) and incubation period, symptoms appear as abdominal cramps, profuse diarrhea, headache, chills, and fever. A large number of pathogens are excreted in the feces. Symptoms can last for 7 to 12 d, but a person can remain a carrier and shed the pathogens in feces for a long time.[16,17]

3. Food Association

Only humans are known to be the host of the pathogen, and a food can get contaminated directly or indirectly though fecal contamination. Outbreaks from the ingestion of foods contaminated with the pathogen have been recorded. The 1971 outbreak in the U.S. from the ingestion of an imported cheese was traced to contamination of the processing plant equipment from a malfunctioning water filtration system. In 1983, another outbreak on a cruise ship was related to potato salad contaminated by a carrier food handler.[16,17]

4. Prevention

The pathogen is sensitive to pasteurization temperature. Thus, proper heat treatment, elimination of postheat contamination for a ready-to-eat food, and refrigeration of a food soon after preparation are necessary to control the disease. In addition, proper sanitation at all stages of food processing and handling is an important factor. Individuals suspected of being carriers should not handle food, especially ready-to-eat food.[16,17]

C. Gastroenteritis due to EHEC

1. Characteristics

The principal serotype associated with enterohemorrhagic colitis is *Esc. coli* O157:H7. As opposed to other *Esc. coli*, it does not ferment sorbitol or have glucuronidase activity. Like other *Esc. coli*, it grows rapidly at 30 to 42°C, grows poorly at 44 to 45°C, and does not grow at 10°C or below. Strains resistant to pH 4.5 or below have been identified. The organism is destroyed by pasteurization temperature and time and killed at 64.3°C in 9.6 sec. The cells survive well in food at –20°C.[17–21]

2. Toxins

Esc. coli O157:H7 produces a verotoxin (VTI), or Shiga toxin (ST). For this, it is also designated as VTEC or STEC. More than one toxin can be involved in the disease and the symptoms related to it. It is not known whether the pathogen also produces invasive factors. The cells probably colonize in the intestine by adhering to the epithelial cells and produce toxins, which then act on the colon. Toxins are also absorbed into the bloodstream and damage the small blood vessels in the intestine, kidneys, and brain.[19]

3. Disease and Symptoms

Esc. coli O157:H7 causes hemorrhagic colitis, hemolytic uraemic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). Symptoms occur 3 to 9 d after ingestion and generally last for 4 d. The colitis symptoms include a sudden onset of abdominal cramps, watery diarrhea (which in 35 to 75% of cases turns to bloody diarrhea), and vomiting. Fever may or may not be an associated symptom. Damage to the lining of the large intestine causes bleeding. Toxins also cause breakdown of red blood cells, and clotting in small blood vessels of the kidney, causing kidney damage and occasional kidney failure, causing HUS. It can be fatal, particularly in children. TTP results from a blood clot in the brain, with seizures, coma, and often death.[17–21]

4. Food Association

The pathogen is probably present in the intestine of animals, particularly in dairy cattle, without producing symptoms. Food of animal origin, especially ground beef,

has been implicated in many outbreaks in the U.S., Europe, and Canada. The affected people were found to have consumed improperly cooked, contaminated hamburgers. In a 1993 outbreak, affecting over 500 people and causing 4 deaths, consumption of hamburgers served by a fast-food chain in Washington, Nevada, Oregon, and California was implicated. The hamburgers, contaminated with *Esc. coli* O157:H7, were cooked at a temperature that failed to kill the pathogen. In addition to ground beef, other foods, such as raw milk, apple cider, some fruits, uncooked sausages, sprouts, and salad have been implicated. Investigations revealed the presence of *Esc. coli* O157:H7 in many different types of foods of animal origin, such as ground beef, pork, poultry, lamb, and raw milk, in low percentages. The organism was isolated in low frequencies from dairy cows as well as calves and chickens.[17–21]

5. Prevention

Proper sanitation, cooking or heating at appropriate temperatures, proper refrigeration, and prevention of cross-contamination should be practiced in order to control the presence of *Esc. coli* O157:H7 in a ready-to-eat food. The Food Safety Inspection Service (FSIS) in the U.S. has provided the following guidelines to control foodborne illness from this pathogen: use only pasteurized milk; quickly refrigerate or freeze perishable foods; never thaw a food at room temperature or keep a refrigerated food at room temperature over 2 h; wash hands, utensils, and work areas with hot soapy water after contact with raw meat and meat patties; cook meat or patties until the center is gray or brown; and prevent fecal–oral contamination through proper personal hygiene.[17–21]

V. SHIGELLOSIS (BACILLARY DYSENTERY) BY *SHIGELLA* SPP.

A. Importance

The genus *Shigella* contains four species: *Shigella dysenteriae, Shi, flexneri, Shi. boydii* and *Shi. sonnei* and each species has several serovars. Only humans and some primates are their hosts. The organisms are either transmitted directly through fecal–oral routes or indirectly through fecal-contaminated food and water. While in most developed countries transmission through drinking water has been reduced, in developing countries contaminated drinking water is a major cause of shigellosis. The disease is prevalent in some geographic locations, particularly in Asia, Mexico, and South America. It occurs more frequently in places with poor sanitation. In the U.S., shigellosis occurs more among migrant workers, on Indian reservations, in poor urban institutions, and in day-care centers. Children below 5 years of age are more affected. In developing countries, there is a high fatality rate among children suffering from shigellosis. In the U.S., before the 1950s, the predominant species was *Shi. dysenteriae*. At present, the predominant species is *Shi. sonnei*. In the U.S. between 1983 and 1987, there were 44 outbreaks affecting 9971 people, with 2 deaths. In one outbreak in 1987, several thousand people were affected by eating commercial meals prepared under poor hygienic conditions that resulted in

contamination of food with *Shi. sonnei*. In general, food service establishments have been implicated in more outbreaks, and poor personal hygiene has been the major cause. The disease is more predominant during late spring to early fall.[12,22]

B. Characteristics

The cells of the species are Gram-negative, nonmotile, facultative anaerobic rods. They are generally catalase positive and oxidase and lactose negative. They ferment sugars, usually without forming gas. On the basis of DNA homology, both *Shigella* and *Escherichia* can be included in one genus, and because of many biochemical similarities, the separation between the two genera is not clear. There are suggestions that *Shigella* species could be pathogenic variants of *Esc. coli*. They are more like Shiga-toxin-producing *Esc. coli*, with *Shigella* O antigen.[23]

The strains grow between 7 and 46°C, with an optimum at 37°C. The cells are not as fragile as once thought. They survive for days under different physical and chemical stresses, such as refrigeration, freezing, 5% NaCl, and pH 4.5. They are killed by pasteurization. The strains can multiply in many types of food when stored at growth temperature range.[22,24]

C. Habitat

The intestine of humans and some primates is the only habitat known. Humans can carry the organism in the intestine, and shed it in the feces without showing any symptoms. Following recovery from shigellosis, an individual can remain a carrier for months.[22,24]

D. Toxins

The strains are believed to carry plasmid-encoded invasive traits that enable the shigellae cells to invade epithelial mucosa of the small and large intestines. Once engulfed by the epithelial cells, they can produce an exotoxin that has an enterotoxigenic property. The toxin is designated as Shiga toxin (ST). The invasive trait is expressed at 37°C but not at 30°C. Shigellae cells growing at 30°C need a few hours of conditioning at 37°C before they can invade intestinal epithelial cells. The engulfed shigellae cells kill the epithelial cells and then attack fresh cells, causing ulcers and lesions.[22,24]

E. Disease and Symptoms

The infective dose is very low, ca. 10^{1-3} cells in adults. Following ingestion of a contaminated food, the symptoms occur in 12 h to 7 d, but generally in 1 to 3 d. In case of mild infection, symptoms last for 5 to 6 d, but in severe cases, symptoms can linger for 2 to 3 weeks. Certain individuals might not develop symptoms. An infected person sheds the pathogens long after the symptoms have stopped. The symptoms are the consequence of both invasiveness of epithelial mucosa and the enterotoxin and include abdominal pain, diarrhea often mixed with blood, mucus

and pus, fever, chills, and headache. Generally, children are more susceptible to the disease than adults.[22–25]

F. Food Association

Shigella cells are present in a food only through fecal contamination, directly or indirectly, from a person either suffering from the disease, or a carrier or a person who has not developed symptoms yet but is shedding the pathogens in feces. Direct contamination occurs from poor personal hygiene. Indirect contamination occurs from the use of fecal-contaminated water to wash foods that are not subsequently heat processed. Also, cross-contamination of ready-to-eat foods can be involved in an outbreak. Foods often implicated in shigellosis are those that are handled too much and are ready-to-eat.

In many developed countries, the most frequently involved foods are different types of salads (potato, tuna, shrimp, and chicken), with potato salads ranked at the top. Foods that are chopped, diced, or cut prior to eating, such as vegetables used in salads, have also been involved in outbreaks. Shellfish harvested from sewage-polluted water and eaten raw have been associated with shigellosis. Many foods support their growth. As the infective dose is very low, growth in food is probably not an important factor for the disease.[22–25]

G. Prevention

Foodborne shigellosis, at least in the developed countries, is caused by contamination of foods by food handlers shedding the pathogen in the feces and having poor personal hygiene. To prevent contamination of ready-to-eat food by such individuals, it is necessary to forbid them to handle such foods. Quite often, this is impossible, especially if the individual is a carrier. Proper education of the food handlers about the importance of good personal hygiene and the need to not handle food if one suspects having a digestive disorder is important. Use of rigid sanitary standards to prevent cross-contamination of ready-to-eat food, use of properly chlorinated water to wash vegetables to be used for salads, and refrigeration of foods are necessary to reduce foodborne shigellosis.[22–25]

VI. CAMPYLOBACTERIOSIS BY *CAMPYLOBACTER* SPP. (*CAMPYLOBACTER ENTERITIS* AND *CAMPYLOBACTER JEJUNI ENTERITIS*)

A. Importance

Several *Campylobacter* species can cause human gastroenteritis; however, *Campylobacter jejuni* and *Cam. coli* are considered the most common causative agents of human diarrheal disease in many countries worldwide. In many countries, the number of cases of campylobacteriosis probably far exceeds the combined number of salmonellosis and shigellosis cases. Epidemiological data have confirmed this in

Canada, the U.K., and Scotland. Isolation of *Campylobacter* spp. from a suspected sample requires specific methods. After developing this method and incorporating it to isolate suspected foodborne pathogens, *Cam. jejuni* has been confirmed as a causative agent in many foodborne illnesses. Since it was first recognized as the cause of an outbreak in 1979, *Cam. jejuni* has been implicated in 53 foodborne outbreaks in the U.S. between 1979 and 1987, affecting 1547 individuals and resulting in 2 deaths. The foods implicated most often in campylobacteriosis were raw milk and improperly cooked chicken. Although several *Campylobacter* spp. have been associated with foodborne campylobacteriosis, *Cam. jejuni* has been isolated in most incidents; the discussion here is on *Cam. jejuni*.[1,2,26,27]

B. Characteristics

Cam. jejuni is a Gram-negative, motile, nonsporulating, rod-shaped bacterium. The cells are small, fragile, and spirally curved. The strains are microaerophilic and catalase and oxidase positive. The strains require a microaerophilic environment of ca. 5% oxygen, 8% CO_2, and 87% N_2 for growth. Growth temperature ranges between 32 and 45°C, with optimum ca. 42°C. They grow better in amino acids than in carbohydrates. They generally grow slowly and are not a good competitor while growing with other bacteria. They do not generally grow well in many foods. They are sensitive to many environmental parameters, including oxygen (in air), NaCl (above 2.5%), low pH (below pH 5.0), temperature (below 30°C), heat (pasteurization), and drying. However, they survive well under refrigeration and for months in the frozen state.[28–30]

C. Habitat

Cam. jejuni is an enteric organism. It has been isolated in high frequency from feces of animals and birds. Human carriers were also found to shed the organisms in feces. Fecal materials from poultry were found to contain $\geq 10^6$ cells/g in some instances. Water, sewage, vegetables, and foods of animal origin are easily contaminated with *Cam. jejuni* excreted through feces.[29,30]

D. Toxins

Cam. jejuni has a thermolabile enterotoxin that is responsible for enteric disease symptoms. The toxin cross-reacts with cholera toxin, and the toxin production trait is plasmid linked. In addition, the strains produce an invasive factor that enables the cells to invade and establish in epithelial cells in both the small and large intestines in humans.[29,30]

E. Disease and Symptoms

The infective dose for campylobacteriosis is considerably low, only ca. 500 cells. Following ingestion, symptoms of the disease occur in 2 to 5 d. Symptoms generally last for 2 to 3 d, but can linger for 2 weeks or more. Persons with no visible symptoms

can shed the cells in feces for a long time. The main symptoms are enteric and include abdominal cramps, profuse diarrhea, nausea, and vomiting. Other symptoms include fever, headache, and chills. In some cases, bloody diarrhea has been reported. An individual can have a relapse of symptoms after a short interval.[29,30]

F. Food Association

Because the organism is present in high frequency in animals, birds, and the environment, many foods, both from plant and animal sources, can be contaminated with *Cam. jejuni*. The foods can be contaminated directly with fecal material from animals and infected humans or indirectly from sewage and contaminated water. *Cam. jejuni* has been isolated at a very high frequency from raw meats (beef, lamb, pork, chicken, and turkey), milk, eggs, vegetables, mushrooms, and clams. In heat-processed food, their presence has been related to cross-contamination following heat treatment or to improper heating. The use of animal feces as fertilizers was found to contaminate vegetables. Outbreaks of campylobacteriosis result from the consumption of raw milk, improperly cooked chicken, dairy products, bakery products, turkey products, Chinese food, eggs, and others. Consumption of raw milk and chicken were implicated in many outbreaks. Although the organism is a poor competitor against other microorganisms present in a food and generally does not grow well in foods, enough cells can survive in a contaminated food to provide the dose required for the disease.[29,30]

G. Prevention

It is rather difficult to control the access of *Cam. jejuni* to raw foods, particularly foods of animal origin. However, proper sanitation can be used to reduce its load in raw foods during production, processing, and future handling. Preventing consumption of raw foods of animal origin, heat-treatment of a food, when possible, and preventing postheat contamination are important to control campylobacteriosis in foods of animal origin. Contamination of vegetables can be controlled by not using animal feces as fertilizer and not using contaminated water to wash vegetables (especially ready-to-eat types). Contamination from humans can be reduced by establishing good personal hygiene and not allowing sick individuals to handle foods, especially ready-to-eat foods.[29,30]

VII. YERSINIOSIS BY *YERSINIA ENTEROCOLITICA*

A. Importance

Foodborne yersiniosis was first confirmed in the U.S. in 1976 following an outbreak among a large number of school children from the consumption of chocolate milk contaminated with *Yersinia enterocolitica*. In many other countries, foodborne yersiniosis was recorded earlier. In Denmark, yersiniosis is one of the most common forms of gastroenteritis. Following the first outbreak in the

U.S., *Yer. enterocolitica* was designated as an emerging foodborne pathogen. Since the first outbreak in the 1970s, several others were recorded until the early 1980s. In 1982, three outbreaks were recorded, one from the consumption of contaminated tofu packed with contaminated spring water and the other two from the consumption of contaminated pasteurized milk. But yersiniosis is not a frequent cause of foodborne infection in the U.S. No incidents were reported between 1982 and 1987, but one was reported in 1988. The two most important aspects about the organism and the disease are that *Yer. enterocolitica* is a psychrotroph and can grow at 0°C, and its symptoms include a sharp abdominal pain with fever, mimicking an appendicitis. *Yer. pseudotuberculosis* is quite common in animals and has been isolated in foods. However, its involvement in foodborne illnesses is confirmed. Only *Yer. enterocolitica* and its association in foodborne illnesses are discussed here.[1,2,31]

B. Characteristics

Yer. enterocolitica cells are Gram-negative short rods, nonsporeforming, motile below 37°C, and facultative anaerobic. The strains grow between 0 and 44°C, with an optimum growth at 25 to 29°C. Growth occurs in milk and raw meat at 1°C, but at a slower rate. Cells can grow in 5% NaCl and at a pH above 4.6. Cells are sensitive to pasteurization.[31,32]

C. Habitat

Yer. enterocolitica is a normal inhabitant of intestines of food animals and birds, pets, wild animals, and humans. Human carriers do not show any disease symptoms. Different types of food can be contaminated from these sources.[31,32]

D. Toxins

Not all strains can produce yersiniosis. Most strains isolated from the environment are nonpathogenic. Pathogenic strains are predominant in pigs. Both the pathogenic and nonpathogenic strains produce a heat-stable toxin; thus, toxin production is not directly related to the ability of a strain to cause yersiniosis. Pathogenic strains also carry an invasive factor that enables the cells to colonize intestinal epithelial cells and lymph nodes. Only after colonization is the heat-stable toxin capable of causing the disease. The pathogenic strains vary in serological characteristics. In the U.S., the most common serovar implicated in yersiniosis is 08.[31,32]

E. Disease and Symptoms

Young children are more susceptible to foodborne yersiniosis. Generally, a high dose (ca. 10^7 cells) is required for the disease. Symptoms are severe abdominal pain at the lower quadrant of the abdomen, diarrhea, nausea, vomiting, and fever. Symptoms generally appear 24 to 30 h following consumption of a contaminated food and last 2 to 3 d. The disease can be fatal in rare cases.[31,32]

F. Food Association

Because *Yer. enterocolitica* strains are found in the environment, many foods can harbor the organism. It has been isolated from raw milk, processed dairy products, raw and improperly cooked meats, fresh vegetables, and improperly chlorinated water. Foods implicated in yersiniosis include raw and pasteurized milk, ice cream, and improperly cooked meats. Because the cells are heat sensitive, a properly pasteurized or heated food can have this pathogen from recontamination following heat treatment. A food can also be contaminated from a human carrier or a pet. As the cells can grow at refrigerated temperature, even a low initial load can reach a high level during extended storage of refrigerated foods.[31,32]

G. Prevention

Because the strains are psychrotrophs, refrigeration cannot be used to control their growth. Good sanitation at all phases of handling and processing and proper heat treatment are important to control the occurrence of yersiniosis. Consumption of raw milk or meat cooked at low temperatures should be avoided.[31,32]

VIII. GASTROENTERITIS BY *VIBRIO* SPP.

In the genus *Vibrio*, four species have been implicated in foodborne illnesses: *Vibrio cholerae* (01 and non-01 serogroups), *Vib. mimicus, Vib. parahaemolyticus*, and *Vib. vulnificus*. *Vib. parahaemolyticus* and *Vib. vulnificus* are discussed in this section and *Vib. cholera* and *Vib. mimicus* are included in the toxicoinfection section. Although there is no clear-cut basis for this differentiation, the absence of fever among individuals affected by both *Vib. cholerae* and *Vib. mimicus* is used to separate the two species from *Vib. parahaemolyticus* and *Vib. vulnificus* infection.[33,34]

A. *Vibrio parahaemolyticus* Gastroenteritis

1. Importance

Gastroenteritis caused by *Vib. parahaemolyticus* is quite common in Japan and accounts for 40 to 70% of the total bacterial foodborne diseases. The high incidence is directly related to the consumption of raw seafoods. In the U.S., its involvement in foodborne infection was first recognized in 1971 from a large outbreak associated with the consumption of steamed crabs contaminated with the pathogen following heat treatment. Several other large-scale outbreaks were confirmed in the 1970s. However, in the 1980s, the number of outbreaks and number of cases per outbreak reduced greatly. Between 1980 and 1987, there were only 12 outbreaks involving a total of 75 people and no fatalities.[33–35]

2. Characteristics

The cells are Gram-negative, nonsporulating, motile, curved rods. They are generally catalase and oxidase positive. The strains grow in a medium containing glucose without producing gas, but are unable to ferment lactose and sucrose. They can grow over a temperature range of 5 to 42°C, with the optimum ca. 30 to 37°C. The cells multiply rapidly in the presence of 3 to 5% NaCl but are sensitive to 10% salt. Under optimum growth conditions, cells can multiply in ca. 15 min. Growth is restricted at pH 5.0 or below. The cells are extremely sensitive to drying, heating (pasteurization), and refrigeration and freezing.[33–35]

3. Habitat

Vib. parahaemolyticus strains are halophilic bacteria distributed in coastal waters worldwide. They are found in estuarine environments and show a seasonal variation, being present in the highest numbers during the summer months. During the winter months, they remain in the estuarine bottom on chitinous materials of plankton.[33–35]

4. Toxin and Toxin Production

Not all strains of *Vib. parahaemolyticus* are pathogenic. The foodborne pathogenic strains can cause hemolysis because of the presence of a heat-stable hemolysin and are designated as Kanagawa-positive. At present, the 23-kDa heat-stable hemolysin (called thermostable direct hemolysin, TDH) is considered to be the toxin. Most strains isolated from natural sources (estuarine water, plankton, shellfish, and finfish) are Kanagawa-negative. However, some Kanagawa-negative strains have also been associated with foodborne outbreaks. All pathogenic strains were found to adhere to human fetal intestinal cells in cell cultures. The toxin production rate and its level are directly related to cell growth, cell concentrations, and pH of the environment. If the toxin forms (in a food), heating does not destroy it.[33–35]

5. Disease and Symptoms

The cells are sensitive to low stomach pH. Generally, an individual has to consume 10^{5-7} cells of a Kanagawa-positive strain for symptoms to develop. However, an increase in pH from consumption of bicarbonates and foods can reduce the infective dose. Symptoms appear 10 to 24 h following ingestion of live cells and last for 2 to 3 d. Symptoms include nausea, vomiting, abdominal cramps, diarrhea, headache, fever, and chills. The disease is not normally fatal.[33–35]

6. Food Association

Vib. parahaemolyticus strains have been isolated in high numbers from various types of seafoods harvested from the estuarine environments, especially during the summer months. The outbreaks, as well as sporadic cases of gastroenteritis, were linked to the consumption of raw, improperly cooked, or postheat-contaminated seafoods,

including fish, oysters, crabs, shrimp, and lobster. In unrefrigerated raw and cooked seafoods, *Vib. parahaemolyticus* can grow rapidly, especially at 20 to 30°C. In temperature-abused seafoods, cells can reach an infective dose level very rapidly, even from a low initial population. Many outbreaks in the U.S. were identified to be due to inadequate cooking and cross-contamination of cooked seafoods, followed by improper holding temperatures.[33–35]

7. Prevention

Several factors need to be considered in controlling gastroenteritis from *Vib. parahaemolyticus*. The seafoods harvested from an estuary should be assumed to contain *Vib. parahaemolyticus*, some strains of which can be pathogenic. In unrefrigerated raw, improperly heated, or postheat-contaminated seafood, the cells can multiply rapidly. Once the pathogenic strains grow to infective numbers, even heat treatment cannot destroy the toxin. With this understanding, the control methods should include the following: no consumption of raw seafoods, proper heat treatment of seafoods, proper sanitation to avoid cross-contamination of heated foods, proper refrigeration of raw and heated products, and consumption of the food within a reasonable period of time. Temperature abuse, even for a short duration, of a seafood should be avoided.[33–35]

B. *Vibrio vulnificus* Septicemia

Vib. vulnificus is a lactose-positive, salicin-positive *Vibrio* species found in the estuarine environment in the coastal waters. It is considered a highly lethal pathogen because of its ability to invade the bloodstream. *Vib. vulnificus* has been associated with fulminating septicemia from the consumption of contaminated seafood, as well as a progressive cellulitis resulting from wound infection. Following consumption of contaminated seafood, in many cases raw oysters, the cells penetrate the intestinal wall and produce primary septicemia in 20 to 40 h. Symptoms are chills, fever, and prostration, with occasional vomiting and diarrhea. The fulminating septicemia sets up rapidly, which can be fatal in many cases. The infection and fatality rate is very high (40 to 60%) among people with liver and gastric diseases and immunodeficiencies. Control measures should include methods discussed for *Vib. parahaemolyticus*. Susceptible individuals should avoid consumption of raw seafoods, such as oysters.[33,34,36]

IX. ENTERIC VIRUSES

A. Importance

Enteric viruses have the potential of becoming a major cause of foodborne illness in the U.S. and most other countries. However, unlike bacterial pathogens, they are difficult to detect and recover from a contaminated food; for some viruses, suitable methods to isolate from a food have not been developed and for some other viruses very little research has been conducted to develop methods to detect them in foods.

Also, unlike bacteria, human enteric viruses do not multiply in food systems; some may die off rapidly under various conditions of food storage and preservation. Thus, seldom is a food routinely examined for contamination by enteric viruses. Even with all the difficulties in confirming a virus as an etiological agent in a foodborne disease outbreak, in the U.S. between 1973 and 1987, viral foodborne infection was the fifth leading cause in both number of outbreaks (5%) and number of cases (9%) of all reported foodborne incidents. Between 1986 and 1987, viral infection ranked third, behind salmonellosis and shigellosis, in relation to total number of cases affected by the foodborne disease outbreaks. During this period, confirmed numbers of outbreaks and cases were 29 and 1067 for hepatitis A and 10 and 1174 for Norwalk-like viruses, respectively. With the improvement in detection techniques, it is anticipated that the incidence of viral foodborne infection will increase. In developing programs to reduce foodborne diseases, this possibility should be considered.[1,2]

B. Characteristics

Foodborne viral infections can occur only from enteric pathogenic human viruses. Before the 1940s, polio viruses transmitted through raw milk were considered the only virus involved in foodborne viral infections. In recent years, hepatitis A (HAV), Norwalk-like viruses (NLV), also called small round structured viruses, (SRVS), Roto viruses, and non-A – non-B hepatitis viruses have been associated with foodborne infections in different parts of the world. Among them, hepatitis A and Norwalk-like viruses are more predominant. Both hepatitis A and NLVs are small (ca. 27 to 28 nm) RNA viruses. Hepatitis A virus can be grown in cell cultures and can be used to produce vaccines. The method to cultivate NLVs in cell cultures has not yet been successful. Their presence in patients' stools is detected by enzyme-linked immunosorbant assay and by nucleic acid sequencing method.[37–39]

C. Habitat

They are of enteric origin and are excreted in very high numbers in human feces. They do not multiply outside the human body, including contaminated foods. Pasteurization can effectively kill the enteric viruses.[37–39]

D. Disease and Symptoms

Enteric viruses can cause infection at a considerably low dose level. Following ingestion of hepatitis A viruses through contaminated food (or other sources), an individual may or may not develop symptoms. In affected individuals, symptoms occur after ca. 4 weeks, with a range of 2 to 7 weeks. The general symptoms are fever, malaise, nausea, vomiting, abdominal discomfort, and inflammation of liver, which may follow with jaundice. Symptoms may last for 1 to 2 weeks or longer. The viruses are shed via feces, generally during the last half of the incubation period. NLVs cause gastroenteritis, characterized by vomiting and diarrhea. Symptoms appear 12 to 24 h after ingestion and last for 1 to 2 d. The viruses are excreted in the feces of infected persons.[37–39]

E. Food Association

Food contaminated with fecal matter of infected people directly (from food handlers) or indirectly (via sewage and polluted water) is the main source of both hepatitis A and NLV outbreaks. Infected food handlers, even without symptoms, can contaminate ready-to-eat food with fecal matter. Vegetables (salads) can be contaminated with polluted water. Shellfish (oysters, clams, mussels, and cockles) harvested from water polluted with sewage and eaten raw or improperly heated before eating have been implicated in many outbreaks of both types of viruses. The virus can survive in shellfish for a long time. Depuration (in tanks filled with disinfected saline water) or relaying (kept in unpolluted water in the sea) for self-cleaning of the viruses from the digestive tracts of the shellfish may not be very effective for hepatitis A and NLVs.[37–39] In the U.S., the major cause of foodborne viral disease outbreaks is the contamination of ready-to-eat foods because of poor personal hygiene and contaminated equipment used with foods served at delicatessens, cafeterias, and restaurants.[1,2]

F. Prevention

The two major preventative methods of foodborne virus infections are to kill the viruses in contaminated foods and adopt good sanitation and personal hygiene habits to control contamination. Proper heat treatment, such as pasteurization, is enough to kill the viruses. Steaming lightly to open the shellfish may not be an effective heat-treatment procedure. Hydrostatic pressure processing, currently being used to open the shellfish, can also destroy the viruses if the pressure used is above 300 MPa. As indicated before, depuration and relaying may not be effective. Sanitation, using oxidative agents such as hypochlorite, can kill viruses in contaminated equipment or in water used in food processing. Good personal hygiene and keeping suspected individuals away from handling ready-to-eat food will also be important to control viral foodborne infections.[37] Vaccination against hepatitis A is available and is used to control the disease.

G. Detection Method

Because enteric viruses do not multiply in food and are usually present in food in relatively low numbers, their detection from a contaminated food poses difficulties. Initially, the viruses are concentrated from a suspected food. For identification of hepatitis A virus, specific cell culture procedures and immunological methods have been developed. The recently developed reverse transcription-polymerase chain reaction (RT-PCR) nucleic acid amplification methods are effective in detecting both hepatitis A and NLVs from shellfish. In one such method, the viral RNA is amplified with hepatitis A or Norwalk-like viral-specific primers by RT-PCR, using a viral RNA internal standard control. By this method, a total of 10^{2-3} viruses were detected in suspected food samples (e.g., ham, turkey, and roast beef).[40]

X. OTHER FOODBORNE INFECTIONS

Several other pathogens are known and confirmed to cause foodborne infections in humans. However, their incidence, especially in outbreaks, is quite low. A brief discussion of some of these diseases and the pathogens is included.

A. Brucellosis

Human brucellosis is caused by *Brucella* spp., namely, *Brucella abortus, Bru. suis*, and *Bru. melitensis*.[41] They are Gram-negative, nonmotile, nonsporeforming, aerobic small rods pathogenic to animals and humans. In infected animals, the organisms are located in the uterus of pregnant animals and in the mammary glands of lactating females. Thus, the pathogens can be excreted in milk. People working with animals can become infected with *Brucella* spp. Also, people working with meat can be infected. Consumption of raw milk and some products made from raw milk (such as some imported cheese) have been implicated in foodborne brucellosis. The cells survive for a long time in milk and milk products. Pasteurization of milk and milk products kills *Brucella* cells. Symptoms of brucellosis in humans include undulant fever with irregular rise and fall of temperature, profuse sweats, body aches, aching joints, chills, and weakness. Symptoms appear in 3 to 21 d following consumption of a contaminated food. Between 1983 and 1987, there were two outbreaks in the U.S. from the consumption of imported cheese affecting 38 people, with 1 death. Control measures for foodborne brucellosis include pasteurization of milk, manufacturing of dairy products from pasteurized milk, and proper sanitation to prevent recontamination of pasteurized products.

B. Streptococcal Infection

Streptococcus pyogenes, in Group A, is a pathogen and has been isolated from lactating animals with mastitis. It is a Gram-positive coccus and has been associated with human pharyngitis with symptoms of sore throat, fever, chills, and weakness.[41] In some cases, nausea, vomiting, and diarrhea can be present. Some strains can cause scarlet fever. Foodborne infections have been recorded from the consumption of contaminated raw milk and milk products made with raw milk and different types of salads (contaminated by infected food handlers). In the U.S., between 1983 and 1987, seven outbreaks affected 1019 individuals. In one outbreak in 1983, 553 people were affected from eating a contaminated potato salad. In addition to Group A *Streptococcus*, some other streptococci have been implicated in foodborne infections. Control measures include pasteurization of dairy products and avoiding the consumption of raw milk and products made with raw milk. People who are suffering from streptococcal infections or are carriers should not handle ready-to-eat foods such as salads. Proper sanitation in all phases of processing and proper refrigeration will help reduce the incidence.

C. Q Fever

Q fever in humans is caused by a rickettsia, *Coxiella burnetii*.[41] Animals carry this organism without any symptom. People handling animals, raw milk, and meat can be infected by the rickettsia and develop symptoms of Q fever. Symptoms include fever, malaise, anorexia, muscular pain, and headache. Symptoms appear 2 to 4 weeks after infection and last for ca. 2 weeks. Foodborne infection occurs from the consumption of raw or improperly pasteurized milk and milk products. *Cox. burnetii* is more resistant to heat than many pathogenic bacteria are. Because of this, the current temperature and time of pasteurization of milk have been set to either 145°F (62.8°C) for 30 min or 160°F (71.1°C) for 15 sec so that *Cox. burnetii* is killed. In the U.S., no incidences of foodborne outbreaks from *Cox. burnetii* have been recorded since 1983.

XI. CONCLUSION

Foodborne infection is caused by several enteric pathogenic bacteria and viruses. The viable cells and particles survive the GI environment and set up infection in the intestine to produce enteric symptoms and fever. Several can also infect the organs inside the body and produce specific diseases. Several pathogens of this group can also produce a high rate of mortality. All of them are sensitive to low heat and killed by pasteurization. Proper sanitation, heat treatment, and refrigeration can be adopted to reduce their incidence. However, a few can multiply even at 1°C; to overcome problems from them, foods should not be refrigerated long, and a refrigerated food should be reheated uniformly before consumption.

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QUESTIONS

1. List some characteristics of foodborne microbial infections. Define enteric and nonenteric infections and give examples of three pathogens in each group.
2. How do symptoms of foodborne infection, intoxication, and toxicoinfection differ?
3. Explain the possible reasons for the current increase in foodborne salmonellosis in a developed country.
4. Briefly discuss the current scheme used in the taxonomy of *Salmonella* serovars.
5. Discuss the regulatory requirements and suggestions directed to control foodborne salmonellosis in the U.S.
6. From the epidemiology of a recent foodborne salmonellosis outbreak (instructor may provide or obtain from the Internet), analyze the causes of the incidence and suggest methods that could be implemented to prevent this.
7. How does foodborne listeriosis differ from foodborne salmonellosis?
8. How can a pasteurized ready-to-eat food be contaminated with *Lis. monocytogenes*? What are the implications of this contamination in a refrigerated ready-to-eat food?
9. List the individuals most susceptible to foodborne listeriosis.
10. "Since 1991 foodborne listeriosis cases have declined." What could have helped in this reduction in the U.S.?
11. Define nonpathogenic and pathogenic *Esc. coli*. How are the pathogenic strains grouped? What are the characteristics of their toxins?
12. Describe the pathogenicity of foodborne EHEC infection in humans.
13. In a 1993 multistate foodborne outbreak, EHEC was isolated from the victims, improperly cooked hamburger patties, and raw hamburger. A large amount of ground beef (several thousand pounds) was suspected of being contaminated with this pathogen. Some food microbiologists believe that when a large volume of a product is contaminated with a pathogen, there is a greater likelihood that a step in the processing operation is directly contaminating the product (which in turn could have been previously contaminated from a small amount of contaminated raw material or environment). With this idea in mind, develop a scenario that might have been involved in the EHEC contamination in large amounts of hamburger patties in the incidence.
14. List two similarities and two differences between foodborne salmonellosis and shigellosis. Define Shinga toxin.

15. Describe food association for a shigellosis outbreak and preventative measures to control the disease. Why can food animals not be the source of *Shigella*?
16. "Campylobacteriosis is emerging as a major foodborne illness in many countries." Justify this statement.
17. Discuss the food association of and preventative measures against the causative pathogen associated with campylobacteriosis.
18. Describe two important characteristics of the causative pathogen associated with foodborne yersiniosis.
19. List the symptoms of yersiniosis and indicate how it is often misdiagnosed.
20. Discuss the control measures to reduce the incidence of foodborne yersiniosis.
21. List the *Vibrio* species considered to be human pathogens.
22. Discuss how foods can be the vehicle of gastroenteritis caused by *Vib. parahaemolyticus*.
23. Describe the pathogenicity of *Vib. vulnificus* in humans.
24. List the enteric viruses implicated in foodborne disease outbreaks. Why are foodborne diseases of viral origin not always detected?
25. How are foods contaminated with hepatitis A virus, and what control measures should be adopted to reduce the incidence?
26. What methods are used to detect incidence of foodborne disease of NLV origin?
27. Briefly describe foodborne infection caused by *Bru. abortus,* pathogenic *Streptococcus*, and *Cox. burnetii*.

CHAPTER 26

Foodborne Toxicoinfections

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I. INTRODUCTION

The pathogenesis and disease symptoms of several pathogens associated with foodborne and waterborne gastroenteritis are somewhat different from classical food poisoning or foodborne infection caused by the pathogens described in Chapter 24 and Chapter 25. Although the differences are not always very clear, in this chapter the gastroenteritis caused by *Clostridium perfringens, Bacillus cereus, Vibrio cholerae*, and enteropathogenic *Escherichia coli* is described as toxicoinfection. The first two are Gram-positive sporeformers, and the last two are Gram-negative small rods. For each pathogen, importance, characteristics, nature of toxins, food association, and control measures are discussed. For some, detection methods and analysis of outbreaks are also included.

The following are some characteristics of foodborne toxicoinfection:

1. For sporeformers, ingestion of large numbers of live vegetative cells is usually necessary.
2. Vegetative cells of sporeformers do not multiply in the digestive tract, but sporulate and release toxins.
3. For Gram-negative bacteria, live cells can be ingested in moderate numbers.
4. Gram-negative cells rapidly multiply in the digestive tract.
5. Many cells also die, releasing toxins.
6. Toxins of both groups produce the gastroenteritis symptoms.

II. *CLOSTRIDIUM PERFRINGENS* GASTROENTERITIS

A. Importance

Gastroenteritis caused by *Clo. perfringens* has several specific characteristics. In most outbreaks, large numbers of cases are involved. In the U.S. in the 1960s and

1970s, it was involved in more than 7% of the total foodborne outbreaks and more than 10% of the total number of cases. In the 1980s, the incidence dropped to ca. 3% of the total outbreaks, affecting ca. 5% of the total cases. The outbreaks generally occur with some foods that were prepared in advance by heating and then kept warm for several hours before serving. In the majority of the instances, these situations are associated with feeding many people within a short period of time in cafeterias, restaurants, schools, and banquets (banquet disease). Between 1983 and 1987, out of 24 outbreaks, 12 were associated with such institutions and only 1 outbreak occurred with food prepared at home. As the disease produces mild symptoms, many incidents are probably not reported. Thus, the reported cases could be only a fraction of the actual numbers.[1,2]

B. Characteristics

The cells are Gram-positive rods, motile, and sporeformers. Cells vary in size and can form small chains. *Clo. perfringens* is anaerobic but can tolerate some air (oxygen). The vegetative cells are sensitive to low-heat treatment (pasteurization), but the spores are extremely heat resistant, and some can survive even boiling for several hours. The cells are resistant to D-cycloserine. In the presence of suitable substrates, H_2S is formed during growth. The cells need several amino acids for growth. Thus, they can grow very effectively in many protein foods. The temperatures of growth of vegetative cells and germination of spores and outgrowth range between 10 and 52°C. The optimum growth occurs at ca. 45°C. At optimum conditions, cell multiplication can be very rapid, in ca. 9 min. The cells fail to grow well at pH <5.0, in NaCl concentration >5%, at A_w, <0.93, and in 500 ppm nitrite.[3–5]

C. Habitat

Spores and vegetative cells are found in soil; dust; intestinal contents of animals, birds, and humans; and sewage. Many foods, particularly raw foods, get contaminated from these sources.[3–5]

D. Toxins and Toxin Production

Among the five types of *Clo. perfringens*, Type A strains are predominantly involved in foodborne toxicoinfection. The enterotoxin associated with the foodborne disease is a heat-labile protein. It is an intracellular protein produced by the cells during sporulation in the intestine and released. Unlike toxins of food-poisoning microorganisms, the enterotoxin is produced in the digestive tract. The environmental parameters for the production of enterotoxin are directly related to the sporulation environment. There are some reports that, in addition to the intestine, sporulation and enterotoxin production to certain levels can also occur in some foods.[3-5]

E. Disease and Symptoms

The enterotoxin causes only gastroenteritis. The symptoms appear 8 to 24 h following ingestion of a large number of viable cells ($\geq 5 \times 10^5$/g) through a food. The main symptoms are diarrhea and abdominal pain. Nausea, vomiting, and fever also can occur but are less common. The toxin changes the permeability of intestinal cells. Fatality, although rare, can occur among the very young, elderly, and sick. Symptoms generally disappear within 24 h. It is considered a mild disease and is seldom reported.[3–5]

F. Food Association

Raw meat from animals and birds is most commonly contaminated with the spores and cells from the digestive tract content, whereas vegetables and spices commonly get them from soil and dust. As the bacterium has some amino acid requirements for growth, meat and meat-containing foods provide good environments for cell growth. The foods commonly incriminated with the outbreaks include meat stews (beef and poultry), roasts, meat pies, casseroles, gravies, sauces, bean dishes, and some Mexican foods (tacos and enchiladas). Table 26.1 lists the food types involved in 190 *Clo. perfringens* gastroenteritis outbreaks in the U.S. from 1973 to 1987. Among the foods listed, protein-rich foods were involved in high percentages.[1] The three most important contributing factors in these outbreaks, in order of importance, were improper holding temperature, inadequate cooking, and contaminated equipment. The most important predisposing cause was cooking food in large volumes in advance, allowing it to cool slowly, then holding for a long period and often serving without reheating. Cooking kills the vegetative cells, but the spores survive. Large volumes of the food provide an anaerobic environment, and slow cooling not only provides the temperature for the spores to germinate and outgrow but also allows cells to multiply rapidly to reach high population levels before eating. The food may not show any loss of acceptance quality.[3]

G. Prevention

The presence of *Clo. perfringens* spores and cells in foods is not uncommon. Unless viable cells are present in high numbers before eating, they cannot cause gastroenteritis; thus, the aim is to keep the cell numbers low. This can be achieved by using proper sanitation in all phases of food preparation and handling. Food should be

Table 26.1 Food Types Involved in *Clo. perfringens* Outbreaks from 1973 to 1987 in the U.S.

Food Types	No. of Outbreaks[a]	%	Food Types	No. of Outbreaks[a]	%
Beef	51	26.3	Finfish	3	1.6
Mexican foods	23	11.9	Shellfish	2	1.0
Turkey	19	9.8	Vegetables	1	0.5
Chicken	9	4.6	Other	46	23.8
Pork	8	4.1	Unknown	28	14.4

[a] Total cases were 12,234.

cooked to the highest temperature recommended to kill the cells and as many spores as possible. The food should be cooled quickly and uniformly (preferably within 1 h) to refrigerated temperature. A shallow container, in contrast to a deep container, facilitates rapid cooling (below 10°C). If a food is stored for a long time, it is important to reheat it quickly and uniformly (to kill vegetative cells) and keep it hot (above 60°C) while being served.[3,5]

H. Detection Method

The detection method involves enumeration of the incriminated food and fecal samples for *Clo. perfringens* in a selective agar medium and incubation of plates under anaerobic conditions.[3,5]

J. Analysis of an Outbreak

In March 1989, 300 of 420 employees became ill after attending a corporate luncheon the previous day. Among the 113 people interviewed, diarrhea, abdominal cramps, and nausea were reported. The symptoms started between 2 and 18 h (mean average 9.5 h) following the luncheon, and the disease lasted ca. 12 h. Stool specimens contained high numbers of *Clo. perfringens*. The menu consisted of chicken, roast beef and gravy, lasagna, and mixed vegetables. Interviews revealed that 98% of those who ate the roast beef became ill; thus, it was implicated as the likely source. Examination of the catering facilities revealed poor sanitation and refrigeration facilities. The roasts, each ca. 19 lb (8.6 Kg), were cooked 3 d before the luncheon date and kept in the refrigerator. Examination of the remaining roast beef showed the presence of *Clo. perfringens* at more than 2×10^6 cells/g.

The roasted meat, prepared in advance under inadequate sanitary conditions, stored at improper temperature, and served without further heating, was associated in this *Clo. perfringens* gastroenteritis outbreak. The most likely sequence of events was as follows. The raw beef (or the facilities in the kitchen) served as the source of the bacterium (most probably spores). Roasting the large-size meat did not kill the spores. Subsequently, during storage for 3 d under improper refrigeration, it took a long time for the temperature in the large roasts to drop below 10°C. Thus, the spores germinated and the cells multiplied rapidly in the protein-rich food and reached a high population before the temperature reached below 10°C. When the roast was served, it had a high number of *Clo. perfringens* cells. Following consumption of the roast, the cells sporulated, releasing enterotoxins and producing the gastroenteritis symptoms in 2 to 18 h, which lasted for ca. 12 h. This outbreak could have been avoided by proper sanitation, proper cooking, proper and quick refrigeration (10°C or below), and warming the roast above 60°C before serving.

III. *BACILLUS CEREUS* GASTROENTERITIS

A. Importance

The incidence of foodborne gastroenteritis by *Bac. cereus* origin is relatively high in some European countries. In contrast, the incidence is quite low in the U.S.,[1,2]

where *Bac. cereus* was recognized as a causative gastroenteritis agent in 1969. Between 1973 and 1987, 58 outbreaks involving 1123 cases were reported. Both the incidence of outbreaks and number of cases per outbreak were low, and no fatalities were reported. However, as the symptoms are not severe and last for ca. 12 h, many cases may not be reported.

B. Characteristics

The cells are Gram-positive motile rods, which form endospores in the middle of the cells. Cells are sensitive to pasteurization. Spores can survive high heat treatment used in many cooking procedures. *Bac. cereus* is aerobic, but can also grow under some degree of anaerobic environment. The cells can multiply in a temperature range of 4 to 50°C, with the optimum ca. 35 to 40°C. Other parameters of growth are pH of 4.9 to 9.3, A_w of 0.95 and above, and NaCl concentration below 10%.[6–8]

C. Habitat

Spores and cells of *Bac. cereus* are common in soil and dust and can be readily isolated in small numbers from many foods, which include both raw and finished products. Intestinal tracts of 10% of healthy adult humans have *Bac. cereus* under normal conditions.

D. Toxins and Toxin Production

The strains produce at least two types (emetic and enteric) of enterotoxins, each probably associated with specific types of symptoms.[3,6] The toxins are produced during growth of cells at the growth temperature range and retained in the cells. Only when the cells are lysed are the toxins released. They occur in the intestinal tract but can also occur in foods. Thus, the cases can also be regarded as food poisoning, as in staphylococcal food poisoning.

E. Disease and Symptoms

In general, a large number of cells ($10^{6–8}$/g) need to be ingested to produce gastroenteritis. The two types of enterotoxins produce two types of symptoms. The enterotoxin associated with the diarrheal form is a heat-labile protein, and that associated with the emetic form is a heat-stable protein. In the diarrheal form, symptoms occur 6 to 12 h following consumption of a food containing the viable cells. Symptoms include abdominal pain, profuse watery diarrhea, and perhaps nausea, but no vomiting or fever. Recovery is usually within 24 h. These symptoms in many respects are similar to those produced by *Clo. perfringens*.

In the emetic form, the symptoms occur 1 to 5 h following ingestion of a food containing the viable cells. As the toxin is heat stable, once the toxin forms in cells, heating food containing a large number of cells before eating can produce the symptoms. Symptoms are nausea and vomiting; abdominal pain and diarrhea may also be present. Symptoms last for ca. 24 h. In some respects, the symptoms are similar to those of staphylococcal gastroenteritis.

F. Food Association

Many types of food can contain small numbers of cells and spores of *Bac. cereus*.[6–8] Consumption of these foods does not cause the disease. However, when these foods are abused to facilitate spore germination and cell multiplication, the population of live cells can reach the high levels necessary for the disease. In the diarrheal outbreaks, a variety of foods, including vegetables, salads, meats, pudding, casseroles, sauces, and soups, has been implicated, mostly because of their improper cooling. However, in the emetic form, outbreaks mostly involve rice and sometimes other starchy foods. Table 26.2 presents a list of foods associated with *Bac. cereus* gastroenteritis outbreaks in the U.S. from 1973 to 1987. Although many foods are listed, the most predominant was fried rice in Chinese restaurants (Chinese rice syndrome). Investigations revealed that, following boiling for preparation, the rice is kept at room temperature for a long time. The surviving (or contaminated) spores germinate, and the cells multiply to high levels before the rice is served.[1,6–8]

G. Prevention

The predominating contributing factors associated with *Bac. cereus* gastroenteritis, in order of relative importance, are improper holding temperature, contaminated equipment, inadequate cooking, and poor personal hygiene. The heat treatment normally used in food preparation, except for pressure cooking, may not destroy *Bac. cereus* spores. The most important control measure is to keep food at a temperature at which the spores do not germinate and cells do not grow. This can be achieved by uniform quick chilling of the food to ca. 4 to 5°C or holding the food above 60°C. Quick chilling can best be accomplished by storing a food in a shallow container, no more than 2 to 3 in. (5 to 6 cm) thick. Because *Bac. cereus* cells, given sufficient time, can grow and produce toxins at refrigerated temperature (≥4°C), a food should not be stored at low temperatures for long periods of time. This means that preparation of a food well in advance should be avoided. Because cells can get in a food through cross-contamination, proper sanitary measures should be adopted while handling a food. Finally, as live cells are necessary for the symptoms, there should be uniform reheating a suspected food to above 75°C before serving. However, heating may not destroy heat-stable toxins associated with emetic symptoms.[3,6]

Table 26.2 Food Types Associated with *Bac. cereus* Gastroenteritis from 1973 to 1987 in the U.S.

Food Type	No. of Outbreaks	%	Food Type	No. of Outbreaks	%
Chinese food	24	41.5	Finfish	1	1.7
Mexican food	5	8.6	Shellfish	2	3.4
Beef	3	5.2	Ice cream	1	1.7
Chicken	1	1.7	Other	8	13.8
Turkey	1	1.7	Unknown	9	15.5
Vegetables	3	5.2			

H. Detection Method

Bac. cereus can be enumerated by surface plating on an agar medium containing mannitol, egg yolk, and polymyxin B (as a selective agent). Colonies surrounded by precipitation resulting from lecithinase of the cells are indicative of *Bac. cereus*.

IV. CHOLERA

A. Importance

Cholera, caused by *Vib. cholerae* 01,[1,2,8,9] is a noncontagious disease but can cause large epidemics with high mortality. In the 19th century, both epidemic and isolated cholera cases were recorded in the U.S. Since 1911, the disease was thought to be eradicated. Most of the outbreaks in the 20th century were recorded in Asian countries. However, in 1973, a cholera case was recorded in Texas. Between 1973 and 1987, 6 outbreaks involving 916 cases with 12 deaths were recorded, mostly in the coastal states of the U.S. Contaminated seafoods (cooked crab, finfish, and raw oysters) were involved in these cases. In 1991 and 1992, a large cholera epidemic that started in Peru spread to many South American countries and affected ca. 640,000 people, with 5600 deaths. It was also introduced into the U.S. by travelers from these countries who either ate contaminated foods before entering the U.S. or brought contaminated concealed foods to the U.S. In addition to the 01 strains, *Vib. cholerae* non-01 strains have also been involved in cholera cases (also designated as non-01 gastroenteritis) in the U.S. and other countries. Non-01 strains were previously thought to be incapable of causing large epidemics, but in 1992, a non-01 serotype, non-0139, was involved in large epidemics in Bangladesh and India.

B. Characteristics

Vib. cholerae, like other vibrios, is a Gram-negative motile, curved rod. The species has many serogroups. Strains in 01 are associated with epidemic cholera. This serotype is further characterized by biotype and serotype. The type currently associated with cholera epidemics worldwide is of the El Tor biotype and either Inaba or Ogawa serotype. Non-01 serotypes do not agglutinate with antibodies prepared against 01 antigens. Also, non-01 serotypes, similar to 01 serotypes, are not sensitive to trimethoprim-sulfamethoxazole with furazolidone. Both types are sensitive to heat and are killed by the temperature used for cooking. Improper heating (at lower temperature for a shorter time) may not be able to kill all the cells present in a food. The optimum temperature of growth is between 30 and 35°C. The growth rate is very rapid, even at room temperature. The cells do not multiply in contaminated live crabs, oysters, or fish. However, in cooked seafoods, rapid growth can occur at 25 to 35°C. Alkaline foods facilitate rapid growth. Survival of cells is better in cooked foods at 5 to 10°C.[9,10]

C. Habitat

Cholera is a human disease. The disease results from the ingestion of infective doses of *Vib. cholerae* cells through food and water contaminated with feces of humans suffering from the disease. Chronic carriers are rare and may not be important in large epidemics. Marine environments may serve as long-term reservoirs. Both serotypes, especially non-01, have been isolated from water in the U.S. Gulf Coast states (Florida, Louisiana, and Texas), in Chesapeake Bay, and along California coasts. Seafoods (crab, oysters, and finfish) harvested from these areas can carry contamination and provide the infective dose. Contaminated water can also be the source of the disease.

D. Toxins and Toxin Production

The toxin of the 01 serotype is a heat-labile, 85-kDa cytotoxic protein with two functional units.[9] The active A subunit stimulates adenyl cyclase in the intestinal epithelial cells, causing massive secretion of water along with chloride, potassium, and bicarbonate in the lining of the intestine. The non-01 serotype produces a cytotoxin and a hemolysin. Following ingestion of *Vib. cholerae* cells in sufficient numbers, the cells colonize the small intestine and multiply rapidly and produce toxins. When the cells die and lyse, the toxins are released into the intestine.

E. Disease and Symptoms

Vib. cholerae is not contagious. A person must consume a large number of viable cells through contaminated food or water to contract the disease. Fecal–oral infection is also possible. The infective dose for cholera is ca. 10^6 viable cells per person, but it varies with age and health. The incubation period ranges from 1 to 5 d, but is usually 2 d. The symptoms include the sudden onset of profuse watery diarrhea and vomiting. Loss of fluid results in dehydration. Other symptoms in severe cases are painful muscle cramps and clouded mental status. Many infected persons may not have any symptom or have mild to moderate diarrhea. Treatments consist of rapid replacement of fluids, along with electrolytes, and administration of proper antibiotics. In addition to diarrhea, the non-01 toxins also cause infection of soft tissues and septicemia.

F. Food Association

Food can serve as a source of *Vib. cholerae* if it is contaminated directly with human feces from the patient or previously contaminated water. The handling of food by a person suffering from the disease can also contaminate food, because of poor personal hygiene. In addition, food originating from natural reservoirs of the causative bacteria can be contaminated and spread cholera. The natural reservoirs include marine and brackish water environments. Testing of samples of water, oysters, crabs, and shrimp from U.S. coastal states has indicated the presence of both 01 and non-01 serotypes. Table 26.3 lists the types of contaminated foods involved in the disease.

Table 26.3 Contaminated Food Associated with Some Cholerae Outbreaks in the U.S.

Food Type	Gulf Coast (1973–1992)		South American Countries (1991–1992)	
	Outbreak	Cases	Outbreak	Cases
Raw oysters	4	4	2[a]	3
Crab[b]	4	32	4	14
Fish and shrimp[c]	1	2	2	77
Cooked rice	1	16	—	—
Seafood[c]	—	—	2	3

[a] In one incident, raw clams were consumed.
[b] Improperly cooked crab.
[c] Some items were raw.

In the Gulf Coast incidents, consumption of raw seafood (oysters and shrimp) and partially cooked crabs (not enough to kill *Vib. cholerae* cells) were mainly involved. These foods were probably contaminated with *Vib. cholerae* cells from the water from which they were harvested. Seafood, eaten either raw or improperly cooked, was responsible for the incidents from South American countries too. These foods either had *Vib. cholerae* when harvested or became contaminated after harvest. One of the most important aspects of this data is to recognize how foods from one country can be involved in a disease in another country.[9]

G. Prevention

The spread of the disease can be prevented or reduced by adopting proper hygienic measures. These include provision for properly treated municipal water, decontamination of other water by boiling or chemical treatment, and proper disposal of sewage. The infected persons may be treated with antibiotics to enhance recovery, along with replacing body fluids. Unexposed people can be vaccinated to protect them from the disease. Seafood should not be harvested from polluted water nor from water found to harbor *Vib. cholerae*. Finally, seafoods should not be eaten raw. The time- temperature of heat treatment of a suspected food should be enough to kill the pathogen. An example of this is the survival of *Vib. cholerae* in contaminated crabs boiled for ca. 8 min; a 10-min boiling time is probably necessary to ensure killing.

H. Detection Method

Isolation of *Vib. cholerae* from a sample is achieved by an initial preenrichment in alkaline peptone water, followed by streaking on a selective agar medium plate (such as thiosulfate citrate bile salt sucrose agar). Suspected colonies (yellow) are biochemically and serologically tested for confirmation. The toxin is detected by immunoassay or bioassay. The toxin gene can be identified by PCR.

I. Analysis of an Outbreak

Between March 31 and April 3, 1991, eight people developed profuse watery diarrhea after eating crabmeat transported from Ecuador; of these, five also had vomiting and

three had severe leg cramps.[11] The symptoms developed 1 to 6 d after consuming the crabmeat. The stool samples of some patients yielded *Vib. cholera*e 01, biotype El Tor, serotype Inaba (the same serotype involved in the cholera epidemic in South American countries, including Ecuador). No crabmeat samples were available for analysis.

Investigations revealed that the crabs were purchased at a fish market in Ecuador, then boiled, shelled, wrapped in foil, and transported by air, unrefrigerated in a plastic bag, to the U.S. on March 30. The meat was delivered to a private residence, refrigerated overnight, and served as a salad on March 31 and April 1.

The probable sequence of events was as follows. The live crabs were contaminated with *Vib. cholerae* harvested from contaminated water, and cooking was not sufficient to kill all the cells. The viable cells subsequently grew in crabmeat at nonrefrigerated temperature to reach the high population necessary to cause the disease. Other possibilities include postheat contamination of the meat during shelling by one or more people with the disease in mild or asymptomatic form, or from the contamination of equipment or the water used. Consumption of the crabmeat caused the disease. Preventing harvest of crabs from contaminated water, proper time and temperature of cooking the crabs, proper sanitation in preparing crabmeat, refrigerating crabmeat following picking, heat treatment before preparing salad — any one or more of these steps could have been used to avoid this incident. (*Note*: It is illegal to bring food into the U.S. from other countries without prior permission of regulatory agencies.)

V. *ESCHERICHIA COLI* GASTROENTERITIS

A. Importance

The two (of the four) enteropathogenic *Esc. coli* subgroups that correlate well with toxicoinfection probably belong to the enteropathogenic and enterotoxigenic *Esc. coli* (EPEC and ETEC, respectively) types. They produce diarrheal diseases when ingested in large numbers through contaminated foods and water. The symptoms are more like those in cholera. The incidence is high in many developing countries and is directly related to poor sanitation.

B. Characteristics

Many serotypes in both subgroups are involved in human gastroenteritis. They are Gram-negative small curved rods, nonsporulating and motile (nonmotile strains can be present). The strains are facultative anaerobes and can grow effectively in both simple and complex media and many foods. Growth occurs between 10 and 50°C, with optimum at 30 to 37°C. Some strains can grow below 10°C. Rapid growth occurs under optimum conditions. Growth-limiting factors are low pH (below 5.0) and low A_w (below 0.93). The cells are sensitive to low-heat treatment, such as pasteurization.

C. Habitat

All strains in both subgroups can establish in the small intestine of humans without producing symptoms. The carriers can shed the organisms in feces and can contaminate food and water directly or indirectly. Many animals, including domestic ones, can also harbor different serotypes of both subgroups and contaminate soil, water, and food. In animals, some serotypes may not produce disease symptoms.

D. Toxins and Toxin Production

The strains in the ETEC subgroup produce two types of enterotoxins: one is heat labile (LT) and the other is heat stable (ST). A strain can produce either LT or ST, or both. LT toxin is an antigenic protein, similar to cholera toxin produced by *Vib. cholerae*, that induces fluid secretion by epithelial cells of the small intestine. ST is a heat-stable protein, lower in molecular weight than LT, and is nonantigenic. It also increases fluid secretion by intestinal cells, but through a different mode of action. Strains in EPEC subgroups were previously thought not to produce enterotoxins such as those by ETEC serotypes. However, some studies have shown that several serotypes produce LT toxin, and several others produce toxins different from LT and ST of ETEC serotypes. In addition to LT and ST enterotoxins, ETEC serotypes can also produce additional factors that enable the cells to colonize, multiply, and initiate infection. The genetic determinants of the enterotoxins in ETEC are plasmid linked and can be transferred to other *Esc. coli* strains.[3,12]

The production of enterotoxins by ETEC strains is influenced by media composition, culture age, and alteration of a culture during growth. Optimum production occurs in a nutritionally rich medium at pH 8.5. Aeration of broth facilitates good toxin production. The toxins are generally detected in a growing culture within 24 h at 35°C. However, the toxin can be produced by the cells growing at 25 to 40°C.[3,12]

E. Disease and Symptoms

EPEC strains were initially thought to be associated with infant diarrhea in many tropical and developing countries, causing high mortality. In contrast, ETEC strains are regarded as the cause of traveler's diarrhea, a nonfatal diarrheal disease. However, some consider that various EPEC strains can also cause traveler's diarrhea. Ingestion of a high level (10^{6-9} cells) of viable cells of the organisms by adults is necessary for the symptoms to occur within 24 to 72 h. Symptoms include mild to severe diarrhea that lasts for 24 to 30 h. In severe cases, dehydration, prostration, and shock may accompany the diarrhea. Not all individuals show symptoms; those who develop symptoms may shed the organisms in feces after recovery.[3,12]

F. Food Association

Many types of foods, including meat products, fish, milk and dairy products, vegetables, baked products, and water have been associated with gastroenteritis of *Esc. coli* origin in many countries.[3,12] These include serotypes from both EPEC and ETEC

subgroups. Direct or indirect contamination of these foods (and water) with fecal materials, along with improper storage temperature and inadequate heat treatment, were involved in these incidences.

G. Prevention

The most important factor in the prevention of gastroenteritis in humans by pathogenic *Esc. coli* is to prevent contamination of food and water, directly or indirectly, by fecal matters. This can be achieved by developing effective sanitation in water supplies and treating and disposing sewage. The other factor is the prevent contamination of food due to poor personal hygiene by people who shed the pathogen. Finally, one needs to recognize that even if the pathogen is present in very small initial numbers in a food, temperature abuse of the food can facilitate multiplication of cells to high levels necessary for the disease symptoms. Thus, food should be refrigerated or eaten quickly, preferably after reheating.[3,12]

H. Detection Methods

The detection methods used involved selective enrichment of sample (food, water, and feces), isolation of pathogens on selective agar medium, and biochemical characterization of suspected isolates. Confirmation tests to detect toxins involve one or more serological tests (ELISA). Other methods to detect toxins include injecting test material into the ligated ileal loop of infant mice or exposing Y-1 adrenal cells to toxin (for LT), or both.

VI. CONCLUSION

Foodborne pathogens in this group include several Gram-positive sporeformers and Gram-negative rods. The cells of sporeformers, when consumed through contaminated foods, sporulate in the gastrointestinal (GI) tract and release the toxin. In contrast, following consumption, Gram-negative rods multiply rapidly in the GI tract and die off, releasing toxins. The symptoms, mainly enteric, are associated with the toxins. They are usually required in very high numbers to cause the diseases. Proper sanitation and refrigeration can be used to reduce the incidence.

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QUESTIONS

1. List and discuss five important factors that differentiate foodborne toxicoinfection from (a) food intoxication and (b) food infection.
2. List the important characteristics of *Clo. perfringens* gastroenteritis (symptoms, suspected foods, and toxin production).
3. Discuss the methods to be adopted to prevent foodborne *Clo. perfringens* gastroenteritis.
4. What are the differences in two types of toxins associated with *Bac. cereus* gastroenteritis? What is the sequence of events that leads to this disease?
5. Discuss the importance of cholera as a foodborne disease. List the various serotypes that are associated with cholera.
6. Discuss the factors and foods involved in the cholera outbreaks in the U.S. since the 1970s.
7. Explain the mechanism of disease production by cholera toxins.
8. Discuss the methods to be implemented to prevent foodborne *Esc. coli* gastroenteritis.
9. Explain the nature of toxins involved in foodborne toxicoinfection by *Clo. perfringens, Bac. cereus, Vib. cholerae* 01 and non-01, EPEC, and ETEC.
10. Explain the terms in relation to foodborne diseases: Banquet disease, Chinese rice disease, seafood disease, traveler's diarrhea, and infant diarrhea.
11. From an outbreak incidence of cholera, EPEC, or ETEC (instructor may provide or get from the Internet), discuss the sequence of events that caused the disease. What could have been done to prevent the incidence?
12. List four factors that could be associated with high incidence of foodborne gastroenteritis, such as traveler's diarrhea in many developing countries.

CHAPTER 27

Opportunistic Pathogens, Parasites, and Algal Toxins

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I. INTRODUCTION

Besides the foodborne bacterial pathogens discussed in Chapter 24 to Chapter 26, several other bacterial species are suspected of having the potential to cause foodborne illness. Normally, they are not pathogenic to humans, but strains in these species have been known to produce toxins. Thus, consumption of foods contaminated with these bacterial species and strains may cause illness, especially under certain circumstances, such as if they are consumed in extremely high numbers or the individuals are either very young or not in normal physical condition. These bacterial species and strains are considered opportunistic pathogens. A brief discussion on the characteristics of some of these species, their association with foods, and their disease-producing potential are discussed in this chapter. A brief discussion is also presented on foodborne illnesses caused by several nonmicrobial components. These include biogenic amines (can be produced by bacterial metabolism in food), algal toxins, and several pathogenic parasites.

II. OPPORTUNISTIC PATHOGENS

A. *Aeromonas hydrophila*

1. Characteristics

The genus includes several species: *Aeromonas hydrophila, Aer. carvie,* and *Aer. sobria*. *Aer. hydrophila* cells are Gram-negative motile rods. The strains are found in both saltwater and freshwater environments and are pathogenic to fish. They are also found in the intestinal contents of humans and animals. Their growth temperature ranges between 3 and 42°C, with an optimum between 15 and 20°C; a few strains can grow at 1°C. They are facultative anaerobes, but grow better in an aerobic environment. Pasteurization effectively kills the cells. Factors such as pH (below 4.5), NaCl (above 4%), and low temperature (below 3°C) can reduce their growth.[1,2]

2. Food Association

Because of the nature of its normal habitat, *Aer. hydrophila* is found in many foods, especially foods of animal origin. It may be isolated from milk, finfish, seafood, red meat, and poultry; in some foods it occurs at a level of 10^5 cells/g or /ml. The strains have been implicated in the spoilage of foods. Because of the psychrotrophic nature, they can grow in foods at refrigerated temperature, and, even from a low initial load, can reach a high population with time during storage. Their presence in high numbers

in food can be controlled by heat treatment, preventing postheat contamination, and using one or more of the growth-limiting parameters, such as low pH and low A_w.[1,3]

3. Disease-Causing Potential

Aer. hydrophila strains have been suspected to cause gastroenteritis in humans, especially when consumed in large numbers and when individuals have impaired health. No confirmed cases of foodborne illness caused by *Aer. hydrophila* have been reported. Several incidences of gastroenteritis from the consumption of food and water contaminated with this species were reported. Many strains of *Aer. hydrophila*, especially those isolated from foods, were found to produce cytotoxins and hemolysins. It is not definitely known whether these toxins can cause gastroenteritis in humans.[1,2]

B. *Plesiomonas shigelloides*

1. Characteristics

Plesiomonas shigelloides is a Gram-negative rod, facultative anaerobic, motile, and nonsporulating. The species has many characteristics similar to those of *Aeromonas* spp. and was previously classified as *Aer. shigelloides*. The organism is isolated from the intestinal contents of humans and warm- and cold-blooded animals. It is found in fresh and brackish water and in fish and oysters harvested from water. Most strains grow between 8 and 45°C, with optimum growth at 25 to 35°C. The cells are killed by pasteurization. Low temperature (below 10°C), low pH (below 4.5), and NaCl (above 5%) can be used, especially in combinations of two or more, to reduce growth.[3,4]

2. Food Association

Ple. shigelloides strains are isolated from foods of aquatic origin, such as fish and shellfish. They are present in higher frequencies and levels in oysters collected during warmer months and from muddy beds. Also, because of fecal contamination, they can be present in raw foods of animal, bird, and plant origin. In heat-treated foods, their presence indicates either improper heating or postheat contamination, or both. They can grow rapidly in most foods under optimum growth conditions. They are not expected to grow in foods held in refrigeration (3 to 4°C or below), even for a long time. Foods with low pH (4.5), low A_w (0.95), and 5% salt discourage their growth.[3,4]

3. Disease-Causing Potential

Ple. shigelloides strains were implicated in many human gastroenteritis outbreaks associated with contaminated drinking water. In many cases, the organisms were isolated from both the implicated water and stools of affected people. The typical symptoms are diarrhea, nausea, abdominal pain; many can also have vomiting, fever,

and chills. The incubation period ranged from 24 to 50 h, and the symptoms lasted for 1 to 9 d. Several incidents of gastroenteritis from the consumption of seafood, oysters, crabs, and fish were suspected to be due to *Ple. shigelloides*. In some instances, the organisms were isolated both from foods and stools of patients. In these cases, foods were eaten either raw or after being improperly cooked or stored.

It is difficult to confirm a direct involvement of *Ple. shigelloides* in human foodborne gastroenteritis. *Ple. shigelloides* was isolated in stools from both unaffected people and individuals suffering from gastroenteritis. Thus, these strains are considered as opportunistic pathogens, affecting individuals with less resistance, such as the young, old, and sick. In addition to gastroenteritis, several strains are associated with bacteriemia and septicemia. Several studies demonstrated that *Ple. shigelloides* strains have heat-stable toxins that may have enterotoxin properties.[3,4]

C. Non-*Escherichia Coli* Coliforms

Coliform groups include species from genera *Escherichia, Klebsiella, Enterobacter*, and *Citrobacter*, all belonging to the family *Enterobacteriaceae* (see Table 29.1), and thus sharing some common characteristics. Previously, *Esherichia coli* strains (both nonpathogenic and pathogenic) were thought to mainly inhabit the intestinal tract of humans and warm-blooded animals and birds, and most species in the other three genera were thought to be mainly of nonintestinal origin. However, other studies have shown that species and strains from *Klebsiella, Enterobacter*, and *Citrobacter* (together referred to as non-*Esc. coli* coliforms) can colonize the human gut and produce potent enterotoxins. In several acute and chronic cases of diarrhea, they were isolated from stools and the intestinal tract. Some isolates of *Enterobacter cloacae, Klebsiella pneumonia*, and *Citrobacter* spp. were found to produce enterotoxins similar to heat-labile or heat-stable toxins of enterotoxigenic *Esc. coli*. In enterotoxigenic *Esc. coli*, these traits are plasmid linked. The ability of non-*Esc. coli* coliforms to produce enterotoxins similar to those of pathogenic *Esc. coli* strains probably results from the intergeneric transfer of plasmids encoding these phenotypes.[5]

Non-*Esc. coli* coliforms are normally present in raw food materials as well as in some pasteurized foods because of postheat contamination. They can grow in many foods if the growth parameters are not limiting. Some strains can grow at refrigerated temperature. Temperature abuse during storage can also facilitate their rapid growth in a food. The significance of their presence in a food may need to be reevaluated. The ability of food isolates to produce enterotoxins may be included in test protocols.[5]

D. Toxigenic Psychrotrophic *Bacillus* spp.

Cells and spores of many *Bacillus* species can be present in non-heat-treated foods. Also, most heat-treated foods can contain their spores. In refrigerated foods, spores of psychrotrophic *Bacillus* spp. can germinate and outgrow, and the vegetative cells can multiply. With time, they can be present in high numbers.

Spoilage of some refrigerated foods has been recognized, such as pasteurized milk, due to growth of several *Bacillus* spp. The psychrotrophic *Bacillus* spp. isolated in one study includes strains of *Bacillus cereus, Bac. mycoides, Bac. circulans, Bac. lentus, Bac. polymyxa,* and *Bac. pumilus*. In this study, an isolate was considered a psychrotroph if it grew at ≤6°C. Many isolates of these species produced toxins. However, the capability of these toxins to cause gastroenteritis was not examined. This aspect may need further consideration as more and more low-heat-processed foods are stored at refrigeration temperature for long times.[6]

Some mesophilic *Bacillus* species, such as several *Bac. licheniformis* strains, are toxigenic and are suspected to have the potential to cause foodborne disease, especially in foods stored or temperature abused at ≥10°C.[7]

III. BIOGENIC AMINES

Different types of amines can form in many protein-rich foods by the decarboxylation of amino acids by the specific amino acid decarboxylases of microorganisms.[8–10] Some of these amines are biologically active and cause illness when consumed in considerable amounts. Examples of biogenic amines are histamine (produced from L-histidine) and tyramine (produced from L-tyrosine). Many bacteria found in foods can produce very active histidine or tyrosine decarboxylases, and, if free L-histidine and L-tyrosine are present, can convert them to histamine and tyramine, respectively. Some food spoilage bacteria and even several lactobacilli can have very active histidine and tyrosine decarboxylases. The protein-rich foods associated with biogenic amine poisoning include cheeses ripened for a long time, mold-ripened cheeses, fermented sausages stored for a long time, and different types of fish. Histamine poisoning of fish origin is further discussed here.[8–10]

A. Histamine (Scombroid) Poisoning

Histamine poisoning is called scombroid poisoning, as the disease was initially recognized to occur from the ingestion of spoiled fishes of scombroid groups (tuna, mackerel, and bonito).[9] However, it is now recognized that the illness can occur from the consumption of other types of spoiled fish, such as mahi mahi, bluefish, yellowtail, amberjack, herring, sardines, and anchovies. Most of these fish have a high level of free L-histamine in their tissues. In addition, the breakdown of muscles by autolytic proteases and bacterial proteases produced during their growth in fish release L-histidine. Histidine decarboxylase produced by bacteria growing in fish catalyzes L-histidine to histamine. The most important bacterial species associated with histamine production in fish is *Morganella morganii*. Several other species are also found, namely, *Kle. pneumoniae, Proteus* spp., *Ent. aerogenes, Hafnia alvei,* and *Vibrio algenolyticus*. In fermented fish products, several *Lactobacillus* spp. have also been implicated in histamine production.[9]

The symptoms of histamine poisoning include gastrointestinal (GI) (nausea, vomiting, abdominal cramps, and diarrhea), neurological (tingling, flushing, palpitations, headache, burning, and itching), cutaneous (hives, rash), and hypotension. Generally, high levels of histamine in fish are necessary to produce the symptoms.

The FDA hazard action level is currently set at 500 ppm in fish. Depending on the histamine concentrations and susceptibility of an individual, symptoms can occur very quickly, generally within 1 h. Histamine poisoning is not fatal. However, the reactions can be severe in individuals allergic to histamine.[9]

Histamine poisoning from fish is a worldwide problem. The frequency of incidence is probably directly related with the popularity of fish and the methods used in preservation from harvest to consumption. In the U.S., between 1973 and 1987, there were 202 incidents affecting 1216 people. The frequency of incidence seemed to increase, as there were 83 incidents between 1983 and 1987. Finfishes were involved in 81 of 83 incidents; 62.6% occurred with fish served in restaurants, and 26.5% occurred at home. The most important factor was improper holding temperature of fish (which caused the fish to decompose). Seasonal variation is not apparent, probably because fish is eaten in the U.S. throughout the year.[9]

As the consumption of fish increases, the incidence of histamine poisoning is expected to rise. The most important control measure will be to store fish by freezing or by refrigeration at <1°C for a limited time. In addition, proper sanitation to reduce contamination with histamine-producing bacteria will be important. Cleaning fish soon after catching can also reduce the autolytic effect of muscle tissues. Histamine is heat stable; once formed, cooking cannot inactivate it. Thus, if there is a doubt that a fish is spoiled, the best way to avoid histamine poisoning is to not eat it.[9]

IV. ALGAL TOXINS

Finfish and shellfish feeding on several species of toxic algae can accumulate toxins. Consumption of these fish and shellfish can produce disease symptoms.[8–10]

A. Ciguatera Poisoning

Finfish, especially those feeding on reef and mud (such as sea bass, grouper, and snapper), while feeding on the toxic algae *Gambierdiscus toxicus* and several others, accumulate toxin in the muscle tissues. Liver, intestines, and roe accumulate more toxin than muscle tissues. Consumption of these fish results in disease, with gastrointestinal and neurological symptoms developing within a few hours. The gastrointestinal symptoms are nausea, vomiting, cramps, and diarrhea, and last for a short time. Neurological symptoms include tingling and numbness of lips and tongue, dryness of mouth, chills, sweating, blurred vision, and paralysis. The disease can be fatal under severe conditions.

Foodborne disease due to ciguatoxin occurs in many countries, especially where reef fishes are consumed. In the U.S., between 1973 and 1987, there were 234 confirmed incidents affecting 1052 individuals, without any fatalities; 232 were from the consumption of finfish harvested from unsafe places. Among the 87 incidents between 1983 and 1987, 69 occurred at home and only 4 occurred in food service establishments.

No method exists at present to detect ciguatoxin, and therefore monitoring fish for the presence of this toxin is not possible. Incidence of the disease can be reduced by avoiding consumption of large fish from the reef, particularly their inner organs.[8–10]

B. Shellfish Poisoning

1. Paralytic Shellfish Poisoning

Scallops, clams, and mussels feeding on *Gonyaulax catenella* and related toxic species of algae accumulate heat-stable toxin in the tissues. Consumption of these shellfish produces paralytic poisoning. The common neurological symptoms include tingling and numbness of lips and fingertips, drowsiness, poor coordination, incoherent speech, and dryness of the throat. In extreme cases, respiratory failure and death can result. The symptoms appear within 1 h and subside within a few days.

Occurrence of paralytic shellfish poisoning is sporadic and depends on the growth (bloom) of the toxic algae, as influenced by water temperature (ca. 8°C), salinity, run-off, presence of nutrients, and others. Shellfish growing in shallow water tend to accumulate more toxins. In the U.S., the incidence of paralytic shellfish poisoning is quite low. Between 1973 and 1987, there were only 21 incidents affecting 160 people. Between 1983 and 1987, only 2 incidents were reported. This has been possible because of monitoring systems of shellfish for toxins and closing contaminated shellfish beds for harvesting.[8–10]

2. Neurotoxic Shellfish Poisoning

Growth of the toxic algae *Ptychodiscus brevis* produces red tide, and feeding of the algae by shellfish causes them to accumulate toxin in the muscle. Consumption of these shellfish produces neurological symptoms as described for paralytic shellfish poisoning, but less severe. The symptoms appear very quickly and generally subside in a few hours. The incidence is very low in the U.S. because of effective monitoring systems.[8–10]

3. Diarrhetic Shellfish Poisoning

Gastrointestinal disorders from the consumption of scallops, mussels, and clams that feed on the toxic algae *Dinophysis fortii* were reported in Japan and the Netherlands. The duration of the symptoms is short. The toxin is heat-stable, and thus cooking does not eliminate it. The incidence of the disease can be reduced by removing the digestive organs of the shellfish, because they tend to accumulate the majority of the toxins.[8–10]

V. PARASITES

Included in this group are several intestinal and tissue helminths (roundworms, flatworms, and tapeworms) and protozoa that are known to cause human illness and where a food association has either been confirmed or suspected. They can stay in the GI tract and produce gastrointestinal symptoms. Some invade body tissues and produce specific problems. Several of these organisms and the diseases they produce are briefly described here.

A. Trichinosis by *Trichinella spiralis*

Trichinella spiralis is a roundworm and can be present in high frequencies in pigs feeding on garbage. It is also found quite frequently among game animals. A person gets infected by consuming raw or insufficiently cooked meat of infected animals. The infected meat contains encysted larvae. Once the meat is consumed, the cysts dissolve in the GI tract, releasing the larvae, which then infect the GI tract epithelium. Here, the parasites mate and deposit larvae in the lymphatic system. Through lymphatic circulation, the larvae infect other body tissues. In the U.S., between 1973 and 1987, 128 incidents of trichinosis infecting 843 people were recorded. Although many types of foods were involved, the highest incidence (55) was from pork. Almost all incidences were due to improper cooking.

The symptoms of trichinosis appear in 2 to 28 d following ingestion. Initial symptoms are nausea, vomiting, and diarrhea, followed by fever, swelling of eyes, muscular pain, and respiratory difficulties. In extreme cases of infection, toxemia and death may result.

The prevention methods consist of not raising pigs on garbage and cooking meat thoroughly [140°F (60°C) and above]. Microwave cooking may not be effective in killing all the larvae. Freezing meat for 20 d at –20°C (home freezer) or in liquid nitrogen for several seconds also kills the parasite.[9,11]

B. Anisakiasis by *Anisakis simplex*

The nematode *Anisakiasis simplex* is a parasite in many fish, particularly of marine origin. Human infection results from the consumption of raw infected fish. Inadequately cooked, brined, or smoked fish has also been implicated in some cases. Symptoms appear after a few days and generally include irritation of throat and digestive tract. Anisakiasis is predominant in countries where raw fish is consumed. Proper cooking, salting, or freezing at –20°C for 3 d can be used as a preventative measure.[12]

C. Taeniasis by *Taenia* spp.

Taeniasis is a tapeworm disease caused by *Taenia solium* from pork and by *Tae. saginata* from beef. The illness results from the ingestion of raw or improperly cooked meat contaminated with larvae of the tapeworms. Symptoms appear within a few weeks and are digestive disorders. If an organ is infected by the larvae, especially a vital organ, the consequences can be severe.[13]

D. Toxoplasmosis by *Toxoplasma gondii*

Toxoplasmosis is a tissue protozoan disease transmitted to humans from the consumption of undercooked and raw meat and raw milk contaminated with oocysts of *Toxoplasma gondii*. Contamination of food with feces, especially from cats, can also be involved. In many people, it does not cause any problems. However, in individuals

with low resistance, the symptoms are generally flu-like, with fever and headache. It can cause fatal deformation and be life-threatening in immunocompromised people. Preventative measures include cooking meat to a minimal internal temperature of 70°C or freezing at –20°C.[14]

E. Giardiasis by *Giardia lamblia*

Giardiasis is produced by the intestinal protozan *Giardia lamblia*. Giardiasis has been considered as food- and waterborne disease in many parts of the world. In the U.S., between 1973 and 1987, five incidents infecting 131 individuals were confirmed. The incidence is much higher in countries with inadequate sanitation facilities and improper water supplies. Contaminated raw vegetables; foods such as salads and sandwiches, contaminated with water containing the causative agent; and poor personal hygiene are considered the major causes of the disease. The main symptoms are acute or chronic diarrhea and abdominal pain. The oocysts and cysts are excreted in the feces of the affected individuals. Wild animals, such as beavers, can carry the protozoa and contaminate water. Improved sanitary conditions and personal hygiene are important to reduce the incidence.[9,15]

F. Cryptosporidiosis by *Cryptosporidium parvum*

Cryptosporidium parvum is an intestinal protozoan and causes cryptosporidiosis in humans through the consumption of contaminated food and water with the oocysts. It can also be present in the GI tract of domesticated and wild animals and contaminate food and water. The major symptoms of infection in humans are watery diarrhea, fever, muscle aches, dehydration, and, in some debilitated individuals, such as AIDS patients, death. Since 1993, there have been four outbreaks in the U.S. from apple cider, chicken salad, and green onions. These foods were contaminated with oocysts by the people infected with the protozoan because they had poor personal hygiene. It is a significant cause of waterborne protozoan disease in humans. The oocysts are resistant to disinfectants normally used to treat water.[15,16]

G. Cyclosporiasis by *Cyclospora cayetanensis*

Cyclospora cayetanensis is a coccidian intestinal protozoan and is considered a newly emerging foodborne pathogen in the U.S. However, the pathogenic nature of the protozoan has been recognized in many other countries for a long time. Humans and some primates are the only natural hosts. Consumption of food and water contaminated with the oocysts and subsequent infection results in the disease. The main symptoms are watery diarrhea, cramps, nausea, vomiting, fever, and fatigue. Since 1995, 80 outbreaks have been reported in the U.S. from the consumption of contaminated raspberries, lettuce, and basil. In one year, contaminated raspberries from Guatemala caused widespread outbreaks in 20 states.[16]

VI. CONCLUSION

Several bacterial species, normally regarded as nonpathogenic, can cause enteric foodborne disease in susceptible individuals. With the rise in susceptible individuals, there may be more cases of foodborne diseases in the future. Increase in consumption of seafood, some as raw, has been regarded as the main cause in the increase of foodborne diseases of algal toxins. Similarly, an increase in the consumption of raw vegetables and fruits has been related to the increase in foodborne diseases caused by different parasites. Proper selection of food, sanitation, and processing and preservation methods can be used to reduce their incidence in food.

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QUESTIONS

1. Define an opportunistic pathogen. Briefly explain how *Aer. hydrophila* can be considered an opportunistic pathogen?
2. Briefly discuss the possibility of causing foodborne gastroenteritis by *Ple. shigelloides*.
3. Name three noncoliforms that may be associated with human foodborne gastroenteritis. What evidence can be given to justify them as the causative agents for gastroenteritis?
4. Discuss the importance of psychrotrophic *Bacillus* spp. as a possible causative agent for foodborne gastroenteritis. Why can the consumption of pasteurized milk stored in the refrigerator for a long time be associated with gastroenteritis caused by psychrotrophic *Bacillus* spp.?
5. Discuss the role of microorganisms in histamine poisoning from the consumption of some ripened cheeses, old fermented sausages, and fish. Name the important microbial species that can be associated with scombroid poisoning. Suggest the preventative methods.
6. Why is ciguatera poisoning more prevalent with foods served at home? How can one prevent the incidents?
7. List the types of shellfish poisoning. What are their major symptoms?
8. List the major causes of trichinosis in humans. How can the incidence be reduced?
9. Discuss the reasons of foodborne anisakiasis, taeniasis, and toxoplasmosis in humans and explain how their incidence can be reduced.
10. List four protozoan diseases transmitted through foods in humans. Discuss the importance of intestinal protozoan diseases of food origin in humans. Which foods are more often associated with these diseases and how can they be reduced?

CHAPTER 28

New and Emerging Foodborne Pathogens

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I. INTRODUCTION

A comparison of the lists of bacteria and viruses that were known to be foodborne pathogens in the past and those confirmed as foodborne pathogens currently reveals quite an astonishing picture. Many that were not considered or known to be foodborne pathogens in the past have later been implicated in foodborne illnesses. These pathogens, following their recognition, are generally designated as new or emerging foodborne pathogens. Table 28.1 presents a list of pathogens recognized as new foodborne pathogens during the last 30 years in the U.S. Before 1959, four bacterial species were considered as foodborne pathogens: *Staphylococcus aureus*; *Salmonella* serovars, including *typhi* and *paratyphi*; *Clostridium botulinum* Types A and B; and *Shigella* spp. In the 1960s, *Vibrio cholerae* non-01 and *Clo. botulinum* Type E were added to the list of bacteria, along with hepatitis A virus. Between 1971 and 1980, *Vibrio parahaemolyticus*, enteropathogenic *Escherichia coli* (O124:B17), *Yersinia enterocolitica, Vib. cholera* 01, *Campylobacter jejuni*, and *Vib. vulnificus* were confirmed either in foodborne disease outbreaks or sporadic cases. The food

Table 28.1 New Pathogenic Bacteria and Viruses Associated with Confirmed Foodborne Diseases from 1959 to 1990 in the U.S.

Before 1959	1959–1970	1971–1980	1981–1990
Sta. aureus	*Clo. perfringens* (1959)[a]	*Vib. parahaemolyticus* (1971)	*Esc. coli* 0157:H7 (1982)
Salmonella serovar (*typhi* and *paratyphi* included)	*Bac. cereus* (1959)	Enteropathogenic *Esc. coli* (0124:B17; 1971)	*Lis. monocytogenes* (1983)
Clo. botulinum (Type A, B)	*Vib. cholera* (non-01; 1965)	*Clo. botulinum* (Infant botulism; 1976)	*Esc. coli* 0127:H20 (1983)
Shigella spp.	*Clo. botulinum* (Type E; mid-1960s)	*Vib. vulnificus* (1976)	Norwalk-like viruses (1982)
	Hepatitis A (1962)	*Yer. enterocolitica* (1976)	Cam. coli
		Vib. cholera (01; 1978)	*Salmonella* Enteritidis[b]
		Cam. jejuni (1979)	

[a] Year first confirmed in foodborne outbreak.
[b] Recognized as a major cause of salmonellosis of egg origin.

association of *Clo. botulinum* with infant botulism was also recognized. In the 1980s, *Listeria monocytogenes*, enterohemorrhagenic *Esc. coli* O157:H7, enterotoxigenic *Esc. coli* O27:H20, Norwalk-like viruses, and probably *Cam. coli* became the new pathogens. *Salmonella* Enteritidis, which was earlier involved in foodborne outbreaks in a relatively low frequency, became a major causative agent of foodborne salmonellosis. In recent years, the possible role of several pathogens, namely, *Helicobacter pyloris*, *Mycobacterium paratuberculosis*, and a few enteric viruses, in foodborne/waterborne diseases has been suspected. Similar situations involving the sudden emergence of new foodborne pathogens with time also exist in other countries.[1–4]

Are they really "new" foodborne pathogens? If a pathogen uses a food as a vehicle for its transmission to humans for the first time and causes the specific illness, then it is probably not new. Rather, it is appropriate to recognize that it probably has caused foodborne illnesses before but has not been confirmed. Because of changes in several situations, its direct involvement in a foodborne illness has probably been suspected before and identified later for the first time. In this section, the changes in several situations that have probably led to the discovery of a new foodborne pathogen are discussed.

A critical analysis of the data in Table 28.1 reveals three important observations: (a) a pathogen that has not been confirmed in the past or at present can emerge as a foodborne pathogen in the future; (b) a pathogen that currently is involved in a few sporadic foodborne illnesses or outbreaks may, in the future, become a major cause of foodborne outbreaks (such as in *Salmonella* Enteritidis); and (c) conversely, a pathogen considered to be the major cause of a foodborne disease in the past or at present can, in the future, become associated with fewer incidences. (For example, in the U.S., there were 26 staphylococcal food intoxication outbreaks in 1976 affecting 930 people; in 1987, there was only 1 outbreak with 100 cases.) The

reasons for these changes can be attributed to many factors, some of which include changes in our food consumption pattern, modern innovation in food processing and retailing technologies, changes in agriculture and animal husbandry practices, increase in food imports from many countries due to changes in the trade barrier, increase in international travel, increase in elderly people and immunocompromised people, and better knowledge of pathogens and regulations for testing foods. Some are discussed here.

II. ASSOCIATED FACTORS

A. Better Knowledge of Pathogens

The etiological agents in foodborne disease outbreaks (and in sporadic incidences) are not always confirmed. Table 28.2 presents epidemiological data of foodborne diseases (bacterial, viral, parasitic, and chemical) from four countries — the U.S. (1960–1987), Canada (1975–1984), the Netherlands (1979–1982), and Croatia (1986–1992). It is surprising that in the U.S., even in the 1980s, the etiological agents for 62% of the outbreaks were not identified (also see tables in Chapter 23 to Chapter 26). Similar situations exist in the other three countries. In general, among etiological agents, bacterial and viral pathogens are the major causes of foodborne illnesses; many of these with unknown etiology are considered to be of bacterial or viral origin. In a reported outbreak, when the food and environmental samples and samples from the patients are available, they are examined for the presence of the most likely or most common pathogens. Thus, in many incidents, the samples will usually not be tested for pathogens that are not considered at that time to be a foodborne pathogen. This is generally the situation with reported sporadic cases or small outbreaks. In case of a large outbreak or a similar type of outbreak occurring

Table 28.2 Total Foodborne Disease Outbreaks of Unknown Etiology in Several Countries

Period (Country)	Outbreaks[a]		Unknown Etiology (%)		Ref.
	Average/Year	Range	Average/Year	Range	
1960–1969[b] (U.S.)	240	91 to 369	44	13 to 60	1
1970–1979 (U.S.)	409	301 to 497	63	55 to 71	3
1980–1987 (U.S)	531	387 to 656	62	54 to 66	3, 5, 6
1975–1984 (Canada)	867	647 to 1180	78	61 to 84	8
1979–1982 (The Netherlands)	292	163 to 415	74	71 to 77	9,10
1986–1992 (Croatia)	49	72 to 37	87[c]	Not available	11

[a] Includes outbreaks of bacterial, viral, parasitic, and chemical origin.
[b] May include a few that were known. In 8 out of 10 years, % unknown ranged from 46 to 60.
[c] For total number of cases.

frequently, or if the incidence results in death or severe consequences, the samples are generally tested for other suspected pathogens along with the common foodborne pathogens. Most of the new foodborne pathogens were discovered this way. In the U.S., the association of *Yer. enterocolitica, Cam. jejuni, Lis. monocytogenes,* and *Esc. coli* O157:H7 in foodborne outbreaks has been confirmed this way. In the future, other pathogens will be recognized as "new" pathogens in the same way. This is probably true for several pathogens, such as *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and other opportunistic pathogens, as well as *Hel. pyloris*, *Myc. paratuberculosis*, and several enteric viruses, which have been suspected from circumstantial evidence to be foodborne pathogens but have not yet been directly confirmed.

One of the major reasons for testing available food samples for new pathogens, other than those tested routinely, is our current expanded knowledge about the characteristics of many pathogens. Much information is available about different pathogens with respect to their physiological, biochemical, immunological, and genetic characteristics, as well as their pathogenicity, habitat, and mode of disease transmission. Recent knowledge on entire genome sequences of several pathogens and advances in molecular biology techniques have greatly aided these studies. This information has helped develop new, effective, and specific methods to isolate and identify foodborne pathogens from samples. Many of the identification techniques are specific, rapid, and less involved, even in the presence of large numbers of associated bacteria. This aids in testing, economically and effectively, a large number of samples in a relatively short span of time for many pathogens, which includes those routinely tested and those that are probably suspected. Availability of ready-to-use kits from commercial sources has advanced this approach.

B. Improvement in Regulatory Actions

In recent years, at least in most developed countries, the local, state, and federal regulatory agencies have been highly active in reporting a foodborne outbreak and taking quick actions to identify the etiological agents.[4] The number of reported outbreaks has increased in the U.S. from an average of 240 per year in the 1960s to an average of 530 per year in the 1980s. This increase is due, in large part, to an increase in reporting. Once a new foodborne pathogen is identified, its frequency of occurrence in foods, mode of transmission in foods, and growth and survival in foods under conditions of processing, storage, and handling at different stages from production to consumption are determined. After this information becomes available, regulatory agencies develop effective methods to either prevent or reduce its presence in the food ready for consumption. This is accomplished by developing proper methods of sanitation during processing and handling of foods and preservation. For some pathogens, methods to reduce their presence in raw foods or even in live animals (such as for *Esc. coli* O157:H7) are being studied. Finally, some improved efforts have been and are being made by regulatory agencies to educate consumers (especially at home) and food handlers (especially at food service facilities) about the means by which one can reduce the incidence of contamination (such as for *Salmonella* from uncooked chicken) due to cross-contamination and improper cooking or cooling of foods. Public education will need increased focus in order to reduce

the incidence of foodborne diseases by pathogens, including those that are and will become newly emerging. Because foods served at home and at food service facilities have been implicated in high frequency in foodborne disease outbreaks, including those with unknown etiology, people involved in handling food in these two places need to be aware of the means by which the incidences can be prevented or reduced.

The efforts by regulatory agencies have probably paid off for several pathogens in the U.S. In the 1980s, as compared with the 1970s, foodborne disease outbreaks from *Sta. aureus* and *Clo. perfringens* were greatly reduced (Table 28.3). In both Croatia and Canada, the incidence of staphylococcal food intoxication is still quite high. The high incidence of outbreaks from *Cam. jejuni* in the U.S. and the Netherlands (also *Yer. enterocolitica*) could be because of testing foods for the presence of the two pathogens on a regular basis in these two countries as compared with in Croatia and probably in Canada. In all four countries, salmonellosis is the most prevalent cause of foodborne disease. This is probably because of the presence in high frequency of *Salmonella* in foods of animal origin and abuse and improper handling of foods at home, in food service establishments, and institutionalized feeding places. The high incidence of shigellosis in the U.S. is probably because of the same reasons. In the U.S., the incidence of botulism is quite high, although the total number of cases is low. Improper home canning of vegetables and foods of animal origin has been the major cause of botulism outbreaks.

Current efforts to identify the incidence of foodborne diseases by establishing FoodNet and proper education of consumers and food handlers by regulatory agencies in the U.S. will be important factors in reducing the incidence of foodborne diseases by currently known pathogens and new pathogens of the future. Similar efforts should be undertaken in other developed and developing countries to overcome problems associated with foodborne pathogens.

C. Changes in Lifestyle and Food Habits

In this category, several factors can be included that have contributed to the emergence of new foodborne pathogens. One of the most important factors is the increase in traveling, especially international traveling. A person arriving at a foreign country

Table 28.3 Ranking of Major Foodborne Pathogens in Four Countries

Rank (No. of Outbreaks)	U.S. (1970s)	U.S. (1983–1987)	The Netherlands (1979–1982)	Canada (1975–1984)	Croatia (1986–1992)
1	*Salmonella*	*Salmonella*	*Salmonella*	*Salmonella*	*Salmonella*
2	*Sta. aureus*	*Clo. botulinum*	*Cam. jejuni*[a]	*Sta. aureus*	*Clo. perfringens*
3	*Clo. botulinum*	*Sta. aureus*	*Yer. enterocolitica*[a]	*Clo. perfringens*	*Sta. aureus*
4	*Clo. perfringens*	*Shigella* spp.	*Clo. perfringens*	*Bac. cereus*	
5	*Shigella* spp.	*Cam. jejuni*			

[a] *Cam. jejuni* and *Yer. enterocolitica* (along with *Vibrio* spp. and pathogenic *Esc. coli*) were reported for the first time.

or returning from it can bring a new foodborne pathogen into a country where it was not recognized previously. *Esc. coli*, associated with traveler's diarrhea, and *Vib. cholerae* non-01 (new serotype) were probably introduced to the U.S. this way.[1–4,15]

Another important factor is changes in food habits. In the U.S., increased consumption of seafoods, some of which are eaten raw, have resulted in an increase in foodborne disease outbreaks by *Vib. parahaemolyticus, Vib. vulnificus, Vib. cholerae,* and Hepatitis A, all of which were not always recognized as foodborne pathogens. Similarly, consumer preferences for low-heat-processed foods with a long shelf life at refrigerated temperatures has enhanced the chances of psychrotrophic pathogens (e.g., *Lis. monocytogenes* and *Yer. enterocolitica*) becoming important foodborne pathogens. Several food preferences, such as consumption of raw milk and undercooked hamburgers, provided the right consequences for *Cam. jejuni* and *Esc. coli* O157:H7, respectively, to cause foodborne disease outbreaks. Similarly, preference for some imported foods, especially the ready-to-eat type, can introduce a new pathogen. An example is the incidence of enterotoxigenic *Esc. coli* O27:H20 from the consumption of a variety of imported contaminated cheeses in the U.S.[1–4,15]

How a change in food consumption patterns can change the profile of foodborne diseases is best exemplified by an increase in foodborne diseases from the increase in consumption of minimally processed ready-to-eat foods. According to a survey by Information Resource, Inc., convenience food continues to be the priority for U.S. consumers. The data show the food preference as follows: ready-to-eat, 77%; heat-and-eat, 76%; packaged for on-the-go consumption, 65%; and no utensils required, 64%. In addition to these, the preference for foods that are more natural has encouraged the production of large varieties of minimally processed foods. Many of these are fresh or given a low-heat treatment and expected to have a shelf life of 3 to 10 weeks or more at refrigerated temperature. Chapter 20 gives examples of many psychrotrophic bacteria being associated with the spoilage of minimally processed foods. Most of these spoilage bacteria gain access to the products as postheat contaminants, generally in low number; during long storage, they multiply to reach the spoilage detection levels. Many of them are new spoilage bacteria or bacteria not previously suspected to be spoilage bacteria. A similar situation can exist for the presence of pathogens in minimally processed foods. The storage temperature of these products ranges from 4 to 15°C or more (during display). Psychrotrophic and many mesophilic pathogens can multiply during storage and from a very low contamination level (e.g., ca. 1/25g) reach to hazard potential level.

Since 1990, there has been an increase in consumption of ready-to-eat raw vegetables (salads), and fruits (fruit juices). Surprisingly, foodborne disease incidence from these sources has increased (Table 28.4).[16,17] Before the 1990s, many of these vegetables and fruits were not considered to be associated with so many different types of foodborne diseases, many of which are thought to be caused by emerging pathogens. Large quantities of this produce come from many countries; often it is difficult to determine the source of production because of complexities involved in international trade. The major sources of contamination are the lack of field sanitation, use of poor-quality water for washing, transportation in dirty trucks, and lack of sanitation in handling the produce.[16,17] Controlled studies showed that many pathogens can not only survive but also grow in fresh vegetables and fruit and

Table 28.4 Fresh Produce Associated with Some Foodborne Diseases in Recent Years in the U.S.

Vegetables	Pathogen	Fruits	Pathogen
Sprouts	*Salmonella, Esc. coli* O157:H7	Cantaloupe	*Salmonella*
Lettuce	*Esc. coli* O157:H7, *Shigella* spp., Hepatitis A virus	Watermelon	*Salmonella,* Norwalk-like virus
Cabbage	*Lis. monocytogenes*	Strawberries	Hepatitis A virus, *Cyclospora*
Celery	Norwalk-like virus	Raspberries	Hepatitis A virus
Carrots	Enterotoxigenic *Esc. coli*	Apple cider	*Esc. coli* O157:H, *Salmonella, Cryptosporidium*
Tomatoes	*Salmonella,* Hepatitis A virus	Apple juice	*Esc. coli* O157:H7
Scallions	*Shigella* spp.	Orange juice	*Salmonella*
		Coconut milk (frozen)	*Vib. cholerae* 01

fruit juices at refrigerated and ambient temperature and many of the pathogens include the emerging type (Table 28.5).

Many minimally processed ready-to-eat foods of animal origin, especially low-heat-processed meat products, have been implicated in foodborne disease outbreaks. Because most of the products are refrigerated, the predominant pathogen was found to be *Lis. monocytogenes* (Table 28.6). As these products were given heat treatment (at pasteurization or higher temperature), the pathogens, namely, *Lis. monocytogenes*, *Salmonella*, and *Esc. coli* O157:H7, could not come from the raw meat and other ingredients. They are, in all probability, postheat contaminants. All the products are handled extensively following heating and before repackaging (in most cases). The pathogens contaminated the products during that time from the sources with

Table 28.5 Growth Potential of Pathogens in Vegetable and Fruit Products

Pathogens	Produce (Temperature)
Shigella	Lettuce, melon, cabbage (20 to 22°C)
Salmonella	Tomatoes, melons, sprouts (20 to 23°C)
Esc. coli O157:H7	Melons, cider, lettuce, cucumber (8 to 25°C)
Yer. Enterocolitica	Different produce (4°C)
Lis. monocytogenes	Different produce (4°C)
Bac. cereus[a]	Vegetable purees (broccoli, peas, carrots, and potatoes; 10°C)
Cam. jejuni	Melon
Enterovirus[b]	Different produce (4 to 23°C)

Remaining data dapted from Sagoo, S.K., Little, C.L., Ward, I., Gillespie, I.A., and Mitchell, R.T., *J. Food Prot.*, 66, 403, 2003.

[a] Supplied by Dr. S.R. Tatini (University of Minnesota, St. Paul).

[b] Survived.

Table 28.6 Pathogens Isolated from Minimally Processed (Cooked) Foods of Animal Origin

Products	Pathogens
Roast and corned beef	*Lis. monocytogenes, Salmonella*
Ham and luncheon meat	*Lis. monocytogenes, Salmonella*
Comminuted products: hot dogs, wieners, bratwurst, salami, bologna, sausage, etc.	*Lis. monocytogenes, Salmonella*
Jerky	*Lis. monocytogenes, Salmonella, Esc. coli* O157:H7
Poultry products: whole, fried parts, nuggets, sliced, etc.	*Lis. monocytogenes, Salmonella*
Meat salads: different types	*Lis. monocytogenes, Salmonella*
Fully cooked meat patties	*Esc. coli* O157:H7
Dry and semidry sausages: pepperoni, salami, summer sausage etc.	*Lis. monocytogenes, Salmonella, Esc. coli* O157:H7, staphylococcal enterotoxins
Cheeses: soft, blue-veined mold-ripened	*Lis. monocytogenes*
Seafoods: smoked, salads	*Lis. monocytogenes*

Source: Adapted from Levine, P., Rose, B., Green, S., Ransom, G., and Hill, W., *J. Food Prot.*, 64, 1188, 2001, and Gombas, D.E., Chen, Y., Clavero, R.S., and Scott, V.N., *J. Food Prot.*, 66, 559, 2003.

which the products come in contact. Consumption of the contaminated products can result in foodborne disease by pathogens, many of which are new and emerging.

D. New Food-Processing Technology

Because of economic reasons, one of the objectives of food processors is to produce a product in large volume and at a faster rate in a centralized plant. Handling a food in large volumes has its disadvantages; accidental contamination of the product by a pathogen can cause a foodborne disease outbreak among large numbers of people over a large area. If the outbreak is due to a pathogen previously unrecognized (or recognized from sporadic incidences), the chance of its being isolated and identified is much higher. An outbreak affecting 220 people from the consumption of chocolate milk produced by a processor led to the examination for other possible suspects, along with those routinely tested, and resulted in the identification of *Yer. enterocolitica* as a new foodborne pathogen.[3]

For faster production, equipment may be designed without much prior consideration of possible microbiological problems, particularly with foodborne pathogens. An incident of listeriosis from the consumption of hot dogs contaminated with *Lis. monocytogenes* is an example. *Lis. monocytogenes* strains were isolated from many types of foods, including ready-to-eat hot dogs and other processed meat products. Epidemiological investigations have also suspected that sporadic listeriosis in humans could be caused by the consumption of foods contaminated with *Lis. monocytogenes*, including hot dogs. In 1989, for the first time in the U.S., a case of human listeriosis was linked to eating ready-to-eat hot dogs because the same serotype (1/2a) of *Lis. monocytogenes* was isolated from the person who ate the hot dogs, from the remaining refrigerated hot dogs in the opened package, and from hot dogs from unopened retail packages produced in the same processing facility. Subsequently, regulatory agencies tested products, line samples, and environmental sam-

Table 28.7 Isolation of *Listeria monocytogenes* Serotype 1/2 a from the Product of Environmental Samples in a Processing Plant

Samples Tested	No. Positive/No. Tested
Packaged products[a]: retail	21/42
Line Samples during Product Manufacture	
During stuffing	0/8
After cooking	1/12
After cooling	0/13
After peeling and before packaging	15/21
Environmental Samples (Swabs)	
Cooking room	0/3
Cooler floor	1/1
Cooler rack and elements	0/2
Peeler area	0/3
Conveyor belt	1/1
Other equipment	0/6

[a] The products were cooked at an internal temperature of 75°C for 2 to 3 h. *Lis. monocytogenes* is not expected to survive if the schedule is followed properly. Contamination occurred following heating and peeling and before packaging.

ples from processing facilities to determine the sources of *Lis. monocytogenes* contamination (Table 28.7). Of the seven retail samples from seven lots produced during a 35-d period, six had *Lis. monocytogenes* 1/2a serotype. Among the line samples collected at different phases of production, *Lis. monocytogenes* 1/2a was found in high frequency in products following peeling and before packaging. (Following peeling, the products traveled via the conveyor belt before going for packaging.) Among the environmental samples (swabs) tested, the same serotype was isolated from the cooler floor, but, more importantly, from the conveyor belt that the heat-treated products came in contact with following peeling. From the analysis of the data, the conveyor belt, attached to the peeler, was suspected to be the main source of product contamination. The porous conveyor belt in use at this processing plant is difficult to clean (see Chapter 21). In designing conveyor belts, the possibility of microbiological problems needs to be considered. Use of conveyors that do not have porous belts or large numbers of small jointed segments (such as small links) may help to efficiently sanitize and control microbiological problems.[3,14]

E. Miscellaneous Factors

Several other factors may have contributed to the emergence of new pathogens in foodborne disease outbreaks. Some of these remain speculative, whereas others have been confirmed. One of the possible reasons for the increase in foodborne gastroenteritis by pathogenic *Esc. coli* strains is the ability of pathogenic *Enterobacteriaceae* to transfer plasmids among themselves that encode for toxin production and colonization in the digestive tract. Such a plasmid transfer in the environment from a pathogenic strain into a nonpathogenic strain will enable the new variant strain to establish in the intestine and produce illness, and a food can be a vehicle through which the pathogen can be consumed.

There are some concerns that use of unrestricted antibiotics in animals has provided the antibiotic-resistant pathogens with a better chance of competing with the sensitive microorganisms and establishing themselves in the environment. In the absence of competition, these pathogens can predominate and, through contamination of a food, emerge as new foodborne pathogens. Such an assumption was made against some sanitary practices used in the processing and handling of foods. Some of the practices are designed to overcome problems from pathogenic and spoilage bacteria traditionally suspected to be present in a food. Elimination of these predominant bacteria may allow minor as well as poorly competing bacteria to become significant in the absence of competition and subsequently cause problems. However, this is probably more important in the case of food spoilage bacteria and has been discussed in Chapter 21.

In recent years, consumption of several low-pH foods, such as orange and apple juices, and fermented sausages, has been implicated in foodborne diseases caused by *Esc. coli* O157:H7, *Salmonella*, and *Lis. monocytogenes*. These pathogens are normally sensitive to low pH ($pH \leq 4.5$) and are expected to die-off rapidly during storage at refrigerated temperature. Studies showed that the pathogens isolated from these foods are acid-resistant variants and are thus able to survive well in a low-pH product (see Chapter 9). Stress response can enable pathogens to survive in food through many processing conditions and make the food potentially hazardous.

There is also some speculation that present methods of raising and feeding food animals and birds may have given some pathogens a better chance to become established as carriers in the animals and birds. Food from these sources has a greater chance of being contaminated with these pathogens. The occurrence of *Esc. coli* O157:H7 in hamburger meat prepared from culled dairy cows and the high incidence of *Salmonella* Enteritidis in eggs and chickens could be due, in part, to the way they are raised now.

Finally, people who are immunocompromised can easily become ill from consuming a food contaminated with a pathogen, even at a low dose level. People with normal resistance will have no problem the same food. This is particularly true for opportunistic pathogens. Some, such as *Aer. hydrophila* and *Ple. shigelloides*, are currently implicated indirectly as possible foodborne pathogens. In the future, people with low immunity may be found to become ill from consuming a food contaminated with an opportunistic pathogen, which then will become a new foodborne pathogen.[16,17]

III. CONCLUSION

The designation *new and emerging foodborne pathogen* may be inappropriate, because many of the so-called new pathogens have been contaminating foods for a long time. For one reason or another, their presence in food has not been examined. Several socioeconomic patterns and lifestyles of consumers have increased the chances of such pathogens being involved in foodborne outbreaks. Scientific understanding and advancement have helped to recognize these chances. In the future, more pathogens, especially viruses, will be in this "new" group and foods will have to be tested for them.

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QUESTIONS

1. Define a new foodborne pathogen.
2. List the new foodborne pathogens that have emerged in the U.S. in the last decade.
3. List the possible factors that can help identify a new foodborne pathogen.
4. Discuss the factors that could be involved in the emergence of a new foodborne pathogen.
5. Discuss how international travel could be a factor in the emergence of a new foodborne pathogen. Give an example.
6. Discuss how new technology can be a factor in increasing the chances of identifying a new foodborne pathogen. Give an example.
7. Why are some pathogens that are recognized as major causes of foodborne illnesses in one country no longer found to be the same in another country? Explain with appropriate examples.
8. "Some opportunistic pathogens of today may be confirmed as new foodborne pathogens of tomorrow." Justify the statement with an example.

CHAPTER 29

Indicators of Bacterial Pathogens

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I. INTRODUCTION

All pathogenic microorganisms implicated in foodborne diseases are considered enteric pathogens, except *Staphylococcus aureus, Bacillus cereus, Clostridium botulinum* (except in the case of infant botulism), *Clo. perfringens,* and toxicogenic molds. This means they can survive and multiply or establish in the gastrointestinal (GI) tract of humans, food animals, and birds. A food contaminated directly or

indirectly with fecal materials from these sources may theoretically contain one or more of these pathogens and can thus be potentially hazardous to consumers. To implement regulatory requirements and ensure consumer safety, it is necessary to know that a food is either free of some enteric pathogens, such as *Salmonella* serovars and *Escherichia coli* O157:H7, or contains low levels of some other enteric pathogens, such as *Yersinia enterocolitica* and *Vibrio parahaemolyticus*. The procedures used to isolate and confirm a pathogen from a food involve several steps, take a relatively long time, and are costly. Some of the new tests involving molecular biology techniques require high initial investment and highly skilled technicians. In a modernized, large commercial operation, involving procurement of ingredients from many countries, processing of different products, warehousing, distribution over a large area, and retail marketing, it is not practical or economical to test the required number of product samples from each batch for all the pathogens or even those that are suspected of being present in a particular product. Instead, food samples are examined for the number (or level) of groups or a species of bacteria that are of fecal (enteric) origin, usually present in higher density than pathogens, but usually considered to be nonpathogenic. Their presence is viewed as resulting from direct or indirect contamination of a food with fecal materials and indicates the possible presence of enteric pathogens in the food. These bacterial groups or species are termed *indicators of enteric pathogens*. Although *Sta. aureus, Clo. botulinum, Clo. perfringens,* and *Bac. cereus* can be present in the fecal matters of humans and food animals, they, along with toxigenic molds, are not considered classical enteric pathogens. Their presence in a food is not normally considered to be because of fecal contamination, and the indicators of enteric pathogens are not very effective for the purpose. To determine the presence of these microorganisms and their toxins, specific methods recommended for their detection and identification should be used. The aerobic plate count (APC) or standard plate count (SPC) is not an indicator of possible presence of pathogens. Instead it is an indicator of the microbiological quality of a food as well as a measure of the level of sanitation used in the handling (processing and storage) of a food (see Appendix E).

II. CRITERIA FOR IDEAL INDICATORS

Several criteria were suggested for selecting a bacterial group or species as an indicator of enteric foodborne pathogens.[1–3] Some are listed with brief explanations:

1. The indicator should preferably contain a single species or a few species with some common and identifiable biochemical and other characteristics in order to be able to identify them from the many different types of microorganisms that might be present in a food.
2. The indicator should be of enteric origin, that is, it should share the same habitat as the enteric pathogens and is present when and where the pathogens are likely to be present.
3. The indicator should be nonpathogenic so that its handling in the laboratory does not require safety precautions as required for pathogens.

4. The indicator should be present in the fecal matter in much higher numbers than the enteric pathogens so they can be easily detected (enumerated or isolated) even when a food is contaminated with small amounts of fecal matter.
5. The indicator should be detected (enumerated or isolated) and identified within a short time, easily, and economically, so that a product, following processing, can be distributed quickly, and several samples from a batch can be tested.
6. The indicator should be detected by using one or more newly developed molecular biology techniques for rapid identification.
7. The indicator should be detected (enumerated or isolated) even in the presence of large numbers of associated microorganisms, which can be achieved by using compounds that inhibit growth of associated microorganisms but not of the indicator.
8. The indicator should have a growth and survival rate in a food as that of the enteric pathogens. It should not grow slower or die off faster than the pathogens in a food. If it dies off more rapidly than the pathogen, then, theoretically, a food can be free of the indicator during storage but can still have pathogens.
9. The indicator should not suffer sublethal injury more (in degree) than the pathogens do when exposed to physical and chemical stresses. If the indicator is more susceptible to sublethal stresses, it will not be detected by the selective methods used in the enumeration, and a food may show no or very low acceptable levels of the indicator even when the pathogens are present at higher levels.
10. The indicator should preferably be present when the pathogens are present in a food; conversely, it should be absent when the enteric pathogens are absent. Unless such correlations exist, the importance of an indicator to indicate the possible presence of a pathogen in a food reduces greatly.
11. There should preferably be a direct relationship between the level of an indicator present and the probability of the presence of an enteric pathogen in a food. This will help set up regulatory standards or specifications for an indicator limit for the acceptance or rejection of a food for consumption. For this criterion, it is very important to recognize whether the high numbers of an indicator in a food have resulted from a high level of initial contamination (and a greater chance for the presence of a pathogen) or from their growth in the food from a very low initial contamination (in which case a pathogen may not be present even when the indicator is present in high numbers).

It is apparent that no single bacterial group or species will be able to meet all the criteria of an ideal indicator. Several bacterial groups or species satisfy many of these criteria. The characteristics, advantages, and disadvantages of some of the important and accepted indicator bacterial groups and species (of enteric pathogens) are described here.[1–3]

III. COLIFORM GROUP

A. Coliforms

In the coliform group, coliforms, fecal coliforms, and *Esc. coli* are discussed. They are not separate, as both fecal coliforms (mostly *Esc. coli*) and *Esc. coli* belong to

coliforms. Depending on the situation, food, water, and environmental samples are examined for one or more of the three.

1. Organisms and Sources

The term *coliform* does not have taxonomic value; rather, it represents a group of species from several genera, namely, *Escherichia, Enterobacter, Klebsiella, Citrobacter*, and probably *Aeromonas* and *Serratia*. The main reason for grouping them together is their many common characteristics. They are all Gram-negative, nonsporeforming rods; many are motile, are facultative anaerobes resistant to many surface-active agents, and ferment lactose to produce acid and gas within 48 h at 32 or 35°C. Some species can grow at higher temperature (44.5°C), whereas others can grow at 4 to 5°C. All are able to grow in foods except in those that are at pH ≤ 4.0 (a few that are acid resistant can grow or survive) and $A_w \leq 0.92$. All are sensitive to low-heat treatments and are killed by pasteurization.

They can be present in feces of humans and warm-blooded animals and birds. Some can be present in the environment and contaminate food. Thus, some *Klebsiella* spp. and *Enterobacter* spp. are found in soil, where they can multiply and reach high population levels. Some are found in water and plants.[1,4–6]

2. Occurrence and Significance in Food

Coliforms are expected to be present in many raw foods and food ingredients of animal and plant origin. In some plant foods, they are present in very high numbers because of contamination from soil. Because they can grow in foods, some even at refrigerated temperature, a low initial number can reach a high level during storage. The occurrence of some coliforms of nonfecal origin and their ability to grow in many foods reduce the specificity of coliforms as an indicator of fecal contamination in raw foods. In contrast, in heat-processed (pasteurized) products, their presence is considered postheat-treatment contamination from improper sanitation. In heat-processed foods, their presence (even in small numbers) is viewed with caution. Thus, in heat-processed foods, their specificity as an indicator is considered favorably (more as an indicator of improper sanitation than fecal contamination). Several selective media have been recommended to determine coliform numbers in food samples. These are selective-differential media and differ greatly in their recovery ability of coliforms (see Appendix E). The results are based on the ability of most coliforms to ferment lactose and produce gas and are available in 1 to 2 d. The presence of sublethally stressed or injured cells can considerably reduce the recovery in selective media. Several other factors, such as high temperature of melted agar media in pour plating, high acidity of a food, and presence of lysozymes (egg-based products) in a food, can further reduce the enumeration of stressed cells. Modified detection methods have been developed to recover injured coliforms (see Chapter 9 and Appendix E). Even with some disadvantages, coliforms are probably the most useful and most extensively used indicators.[1,4–7]

B. Fecal Coliforms

1. Organisms and Sources

Fecal coliform bacteria also constitute a group of bacteria and include those coliforms whose specificity as fecal contaminants is much higher than that of coliforms. This group includes mostly *Esc. coli*, along with some *Klebsiella* and *Enterobacter* spp. Nonfecal coliforms are eliminated by using a high incubation temperature (44.5 ± 0.2 or 45.0 ± 0.2°C) for 24 h in selective broths containing lactose. Lactose fermentation, with the production of gas, is considered a presumptive positive test.[1,7]

2. Occurrence and Significance in Food

Some fecal coliforms are present in raw foods of animal origin. They can be present in plant foods from contaminated soil and water. High numbers can be due to either gross contamination or growth from a low initial level, probably because of improper storage temperature. Their presence in heat-processed (pasteurized) foods is probably because of improper sanitation after heat treatment.

In raw foods that are to be given heat treatment, their presence, even in high numbers ($\cong 10^3$/g or /ml) is not viewed gravely; if the numbers go higher, some importance is given to contamination of fecal matter, improper sanitation, and possible presence of enteric pathogens. A need for corrective measures becomes important. In contrast, in heated products and ready-to-eat products (even raw), their presence, especially above a certain level, is viewed cautiously for possible fecal contamination and presence of enteric pathogens. A food can be accepted or rejected based on the numbers present. This group is extensively used as an indicator in foods of marine origin (shellfish) and in water and wastewater.[1,7]

C. *Escherichia coli*

1. Organisms and Sources

In contrast to either coliforms or fecal coliforms, *Esc. coli* has a taxonomic basis.[1] It includes only the *Escherichia* spp. of the coliform and fecal coliform groups. *Esc. coli* strains conform to the general characteristics described for coliform groups. Biochemically, they are differentiated from other coliforms by the indole production from tryptone, methyl red reduction due to acid production (red coloration), Voges Proskauer reaction (production of acetyl-methyl carbinol from glucose) and citrate utilization as a C-source (IMViC) reaction patterns. *Esc. coli* Type I and Type II give IMViC reaction patterns, respectively, of + + – – and – + – –. The – + – – reaction pattern of *Esc. coli* Type II could also be due to slow or low production of indole from tryptone (or peptone). The IMViC tests are conducted with an isolate obtained after testing a food sample for coliform group or fecal coliform group. However, there is a concern now about the adequacy of these reaction patterns to identify *Esc. coli* types.

Initially, *Esc. coli* types were used as indicators of fecal contamination and possible presence of enteric pathogens (in food), with the considerations that they are nonpathogenic and occur normally in the GI tract of humans, animals, and birds in high numbers. However, it is now known that some variants and strains of *Esc. coli* are pathogenic (e.g., *Esc. coli* O157:H7). None of the methods mentioned previously are able to differentiate pathogenic from nonpathogenic *Esc. coli* strains; this can be achieved only through specific tests designed to identify different pathogenic *Esc. coli* strains. This discussion emphasizes the value of *Esc. coli* as an indicator.[1] The significance and importance of pathogenic *Esc. coli* have been discussed previously (Chapter 25 and Chapter 26).

2. Occurrence and Significance in Food

Esc. coli is present in the lower intestinal tract of humans and warm-blooded animals and birds. Its presence in raw foods is considered an indication of direct or indirect fecal contamination. Direct fecal contamination occurs during the processing of raw foods of animal origin and because of poor personal hygiene of food handlers. Indirect contamination can occur through sewage and polluted water. In heat-processed (pasteurized) foods, its presence is viewed with great concern. Its value as an indicator of fecal contamination and the possible presence of enteric pathogens is much greater than that of coliform and fecal coliform groups. However, the time to complete the tests (IMViC) is relatively long (ca. 5 d). Some direct plating methods have been developed that give an indication of *Esc. coli* in a shorter time. There are several other inadequacies of *Esc. coli* as an indicator. *Esc. coli* strains may die at a faster rate in dried, frozen, and low-pH products than some enteric pathogens, and some enteric pathogens can grow at low temperatures (0 to 2°C), at which *Esc. coli* strains can die. In addition, *Esc. coli* strains can be injured by sublethal stresses in higher degrees than some enteric pathogens, and may not be effectively detected by the recommended selective media unless a prior resuscitation (repair) step is included.

IV. ENTEROBACTERIACEAE GROUP

The methods recommended to detect coliforms and fecal coliforms and *Esc. coli* are based on the ability of these bacterial species to ferment lactose to produce gas and acid. In contrast, some enteric pathogens do not ferment lactose, such as most *Salmonella* serovars. Thus, instead of only enumerating coliforms or fecal coliforms in a food, enumeration of all the genera and species in the *Enterobacteriaceae*[1,2] family is advocated. Because this family includes not only coliforms but also many genera and species that are enteric pathogens, enumeration of the whole group can be a better indicator of the level of sanitation, possible fecal contamination, and possible presence of enteric pathogens (Table 29.1). In European countries, this concept has been used to a certain degree. The method includes the enumeration of organisms from colony-forming units in a selective-differential agar medium containing glucose instead of lactose.[1]

Table 29.1 Genera, Habitat, and Association to Foodborne Illnesses of the *Enterobacteriaceae*

Genera[a]	Main Habitat	Associated with Foodborne Illness[b]
Escherichia	Lower intestine of humans and warm-blooded animals and birds	Only the pathogenic strains
Shigella	Intestine of humans and primates	All strains
Salmonella	Intestine of humans, animals, birds, and insects	All are considered pathogenic
Citrobacter	Intestine of humans, animals, birds; also soil, water, and and sewage	Can be opportunistic
Klebsiella	Intestine of humans, animals, birds; also soil, water, and grain	Can be opportunistic
Enterobacter	Intestine of humans, animals, birds; widely distributed in nature, mostly plants	Can be opportunistic
Erwinia	Mostly in plants	No association
Serratia	Soil, water, plants, and rodents	Can be opportunistic
Hafnia	Intestine of humans, animals, birds; also soil, water, and sewage	No association
Edwardsiella	Cold-blooded animals and water	No association
Proteus	Intestine of humans, animals, birds; also soil and polluted water	Can be opportunistic
Providencia	Intestine of humans and animals	Can be opportunistic
Morganella	Intestine of humans, animals, and reptiles	Can be opportunistic
Yersinia	Intestine of humans and animals; also environment	Some species or strains are pathogenic
Obesumbacterium	Brewery contaminant	No association
Xenorhabdus	Nematodes	No association
Kluyvera	Soil, sewage, and water	Can be opportunistic
Rahnella	Fresh water	No association
Tatumella	Human respiratory tract	No association

[a] Some are of nonfecal origin.
[b] The pathogenic species and strains in the indicated genera are confirmed with foodborne and waterborne illnesses. Some species and strains in some genera, indicated as opportunistic, are suspected of being foodborne pathogens.

This concept is criticized because many species in the *Enterobacteriaceae* are not of fecal origin; many are found naturally in the environment, including plants, and those that form typical colonies because of glucose fermentation in the selective medium do not all belong to this family. However, in heat-processed foods (all are sensitive to pasteurization) and ready-to-eat foods, their presence in high numbers should have public health significance.

V. ENTEROCOCCUS GROUP

A. Characteristics and Habitat

The genus *Enterococcus* is relatively new and includes many species that were previously grouped as fecal streptococci and other streptococci.[7–10] They are Gram-positive, nonsporeforming, nonmotile cocci or coccobacilli, catalase negative, and facultative anaerobes. They can grow between 10 and 45°C, and some species can grow at 50°C. Some require B vitamins and amino acids for growth. Some can survive pasteurization temperature. In general, they are more resistant than most coliforms to refrigeration, freezing, drying, low pH, NaCl, and water. They are found in the intestinal tracts of humans and warm- and cold-blooded animals, birds, and insects. Some can be species specific whereas others can be present in humans, warm-blooded animals, and birds. Among the currently recognized species, several are found in the intestine of humans and food animals and birds, including *Enterococcus faecalis, Ent. faecium, Ent. durans, Ent. gallinarum, Ent. avium*, and *Ent. hirae*. Many have been found in vegetation, processing equipment, and processing environments. Once established, they can continue to multiply in the equipment and environment and are often difficult to completely remove. They are found in sewage and water, especially polluted water and mud. They probably do not multiply in water, but can survive longer than many coliforms. They can grow in most foods.

B. Occurrence and Significance in Food

Enterococcus can get in different foods through fecal contamination or through water, vegetation, or equipment and processing environments, and may not be of fecal origin. In this respect, its value as an indicator of fecal contamination and possible presence of enteric pathogens in food is questionable. Also, the ability of some strains to survive pasteurization temperature (being thermodurics) reduces their value as an indicator. On the other hand, their better survivability in dried, frozen, refrigerated, and low-pH foods and water can make them favorable as indicators. Currently, their presence in high numbers, especially in heat-processed (pasteurized) foods, can be used to indicate their possible presence in high numbers in raw materials and improper sanitation of the processing equipment and environment. They have been used to determine the sanitary quality of water in shellfish beds and are considered to be better as indicators than coliforms for shellfish. Some strains have also been associated with foodborne gastroenteritis, probably as opportunistic pathogens.[8–11]

VI. CONCLUSION

In food microbiology, the concept of indicator bacteria was introduced to measure the sanitary quality of pasteurized foods (e.g., milk). Its purpose was to indicate the possible presence of enteric pathogens (e.g., *Salmonella*) in the food. Over time, the

suitability of several bacterial groups has been considered and this aspect has been discussed in this chapter. It is apparent that selection of a suitable indicator and its level may change with food types and for raw, processed, and ready-to-eat food.

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QUESTIONS

1. Discuss the need of using indicator bacteria for enteric pathogens in food.
2. List with brief explanations six criteria that should be considered in selecting an indicator of enteric pathogens.
3. Which foodborne pathogens are generally not considered enteric? Why? What could be done to determine their possible presence in a food?
4. What are some similarities and differences in characteristics between coliforms and fecal coliforms? What is the significance of their presence in high numbers

($\geq 10^4$/g or /ml) and low numbers ($\leq 10^1$/g or /ml) in a raw food, in a pasteurized food, and in a ready-to-eat food?

5. Why are fecal coliforms considered a better indicator of enteric pathogens than are coliforms?
6. What are the advantages and disadvantages of using *Esc. coli* as an indicator of enteric pathogens in food? "An indicator should be present when an enteric pathogen is present in a food." Why might this statement not be valid for *Esc. coli* as an indicator?
7. List the conditions under which coliforms, fecal coliforms, and *Esc. coli* have questionable values as indicators of enteric pathogens.
8. What are the justifications of using the *Enterobacteriaceae* family as indicators? What are the disadvantages of using this group as an indicator of enteric pathogens?
9. List some characteristics of enterococci that put them in an advantageous position over coliforms as indicators of enteric pathogens. What are the disadvantages of using enterococci as indicators?
10. Oyster harvesting water beds in rivers is often closed because of high levels of indicator bacteria. Explain the advantages and disadvantages of using fecal coliforms and enterococci as sanitary indicators in water samples, mud, and oyster meat.

SECTION VI

Control of Microorganisms in Foods

It is apparent from the materials presented in the previous chapters that although some microorganisms are desirable for the production of bioprocessed food, many are undesirable because they can cause food spoilage and foodborne diseases. For efficient production of bioprocessed food, the objectives are to stimulate growth and increase the viability of desirable microorganisms.

In contrast, with respect to spoilage and pathogenic microorganisms, the objective is to minimize their numbers or completely eliminate them from food. Several methods, individually or in combinations, are used to achieve these goals by (1) controlling access of the microorganisms in foods, (2) physically removing the microorganisms present in foods, (3) preventing or reducing the growth of microorganisms and germination of spores present in foods, and (4) killing microbial cells and spores present in foods.

The influence of intrinsic and extrinsic factors necessary for optimum microbial growth and the range in which each factor supports microbial growth have been discussed in Chapter 6. To control growth and kill microorganisms in a food, some of these factors are used, but only beyond the range that supports growth and induces germination of spores but can induce injury and death (Chapter 9). Some of these controlling methods are discussed here.

Irrespective of the methods used, it is important to recognize that a control method is more effective when a food has fewer microbial cells and when the cells are in the exponential growth phase and are injured. Also, spores are more resistant than vegetative cells, and Gram-negative cells are generally more susceptible than Gram-positive cells to many control methods. Finally, bacteria, yeasts, molds, phages, and viruses differ in sensitivity to the methods used to control them. Different species and strains of the same species also differ in sensitivity to these control methods.

The following control or preservation methods are discussed in this section:

Chapter 30: Control of Access (Cleaning and Sanitation)
Chapter 31: Control by Physical Removal
Chapter 32: Control by Heat
Chapter 33: Control by Low Temperature
Chapter 34: Control by Reduced A_w
Chapter 35: Control by Low pH and Organic Acids
Chapter 36: Control by Modified Atmosphere (or Reducing O–R Potential)

Chapter 37: Control by Antimicrobial Preservatives
Chapter 38: Control by Irradiation
Chapter 39: Control by Novel Processing Technologies
Chapter 40: Control by a Combination of Methods (Hurdle Concept)

CHAPTER 30

Control of Access (Cleaning and Sanitation)

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I. INTRODUCTION

The internal tissues of plants and animals used as foods are essentially sterile. However, many types of microorganisms capable of causing food spoilage and foodborne diseases enter foods from different sources. This has been discussed in Chapter 3. It is impossible to prevent access of microorganisms in food from these sources. However, it is possible to control their access to food in order to reduce the initial load and minimize microbial spoilage and health hazard. This is what regulatory agencies advocate and food processors try to achieve through sanitation.

When and how sanitation was introduced in food-handling operations is not clearly known. However, the consequences of changes in food consumption and production patterns during the late 19th and early 20th centuries, and the understanding of the scientific basis of food spoilage and foodborne diseases, may have helped enhance food sanitation. An outcome of the Industrial Revolution was an increased population in cities and urban areas, which needed processed foods for convenience and stable supply. The foods produced by available processing techniques were not safe. With the knowledge of microbial association with food spoilage and foodborne diseases, food-processing techniques to reduce spoilage and ensure the safety of foods were studied. It was recognized during this time that microorganisms can get in food from various sources, but that proper cleaning and sanitation during handling of foods could reduce their levels, and a food with a lower level of microorganisms could be processed and preserved more effectively to ensure stability and safety than a food with a high initial microbial load. Thus, sanitation became an integral part of food-processing operations.

In recent years, more foods than ever are being processed in both developed and developing countries. In addition, particularly in some countries such as the U.S., many centralized processing facilities are producing foods in large volume. In these centralized plants, many types of raw materials and finished products are handled at a rapid rate. This has been possible because of the availability of needed processing technologies capable of handling large volumes. Some of the food contact machineries are extremely complex and automated and require special methods for effective sanitation.

Although our understanding of the mechanisms by which microorganisms contaminate foods and the means by which that can be intervened have increased, the volume of foods spoiled and the incidence of foodborne diseases remain high. This indicates the need for more effective methods to control microbial access to foods through efficient sanitation (see Appendix D: HACCP).

II. OBJECTIVES

The main objective of sanitation is to minimize the access of microorganisms in food from various sources at all stages of handling.[1,2] Because the microbial sources and level of handling vary with each food of plant and animal origin and fabricated foods, the methods by which microorganisms contaminate foods differ.

Proper sanitation helps reduce the microbial load to desired levels in further processed food. An example of this is that a low microbial level in raw milk produced through effective sanitation makes it easier to produce pasteurized milk that meets the microbial standard. Also, proper sanitation helps produce food that, when properly handled and stored, will have a long shelf life. Finally, proper sanitation helps reduce the incidence of foodborne diseases.[1,2]

III. FACTORS TO CONSIDER

To minimize the access of microorganisms in foods, the microbiological quality of the environment to which a food is exposed (food contact surfaces) and the ingre-

dients added to a food should be of good microbiological quality. To achieve these goals, several factors need to be considered, which are briefly discussed here.[1–4]

A. Plant Design

At the initial designing stage of a food-processing plant, an efficient sanitary program has to be integrated in order to provide maximum protection against microbial contamination of foods. This includes both the outside and the inside of the plant. Some elements to consider are specific floor plan, approved materials used in construction, adequate light, air ventilation, direction of air flow, separation of processing areas for raw and finished products, sufficient space for operation and movement, approved plumbing, water supply, sewage disposal system, waste treatment facilities, drainage, soil conditions, and the surrounding environment. Regulatory agencies have specifications for many of these requirements and can be consulted at the initial stage of planning to avoid costly modifications.[1–4]

B. Quality of Water, Ice, Brine, and Curing Solution

Water is used as an ingredient in many foods and is also used in some products after heat treatment. The microbiological quality of this water, especially if the foods are ready-to-eat types, should not only be free from pathogens (as in drinking water) but also be low (if not free) in spoilage bacteria such as *Pseudomonas* spp. This is particularly important for foods kept at low temperature for extended shelf life. The ice used for chilling unpackaged foods also should not contaminate a food with pathogenic and spoilage bacteria. Water used for chilling products, such as chicken at the final stage of processing, can be a source of cross-contamination of a large number of birds from a single bird contaminated with an enteric pathogen. Similarly, the warm water used to defeather chicken can be a source of thermoduric bacteria.

Brine and curing solutions used in products such as ham, bacon, turkey-ham, and cured beef brisket can be a source of microbial contamination. To reduce this, brine and curing solutions should be made fresh and used daily. Storing brine for extended periods before use may reduce the concentration of nitrite through formation and dissipation of nitrous oxide and may reduce shelf life of the products.[1–4]

C. Quality of Air

Some food-processing operations, such as spray drying of nonfat dry milk, require large volumes of air that come into direct contact with the food. Although the air is heated, it does not kill all the microorganisms present in the dust of the air and thus can be a source of microbial contamination of foods. The installation of air inlets to obtain dry air with the least amount of dust and filtration of the air is important to reduce microbial contamination from this source.[1–4]

D. Training of Personnel

A processing plant should have an active program to teach the plant personnel the importance of sanitation and personal hygiene to ensure product safety and stability.

The program should not only teach how to achieve good sanitation and personal hygiene but also monitor the implementation of the program. People with an illness and infection should be kept away from handling the products. Some kind of incentive to the workers might help make the program efficient.[1–4]

E. Equipment

The most important microbiological criterion to be considered during the design of food-processing equipment is that it should protect a food from microbial contamination. This can be achieved if the equipment does not contain dead spots where microorganisms harbor and grow or that cannot be easily and readily cleaned in place or by disassembling. Some of the equipment, such as meat grinders, choppers, or slicers and several types of conveyor systems, may not be cleaned and sanitized very effectively and therefore serve as a source of contamination to a large volume of product. This is particularly important for products that come in contact with equipment surfaces after heat treatment and before packaging.[1–4]

F. Cleaning of Processing Facilities

Cleaning is used to remove visible and invisible soil and dirt from the food-processing surroundings and equipment. The nature of soil varies greatly with the type of food processed, but chemically it consists of lipids, proteins, carbohydrates, and some minerals. Although water is used for some cleaning, to increase efficiency of cleaning, chemical agents or detergents are used with water. In addition, some form of energy with the liquids, such as spraying, scrubbing, or turbulent flow, is used for better cleaning.

Many types of detergents are available, and they are selected based on the need. The effectiveness of a cleaning agent to remove soil from surfaces depends on several characteristics, such as efficiency of emulsifying lipids, dissolving proteins, and solubilizing or suspending carbohydrates and minerals. In addition, a detergent should be noncorrosive, safe, rinsed easily, and compatible, when required, with other chemical agents. The detergents frequently used in food-processing facilities are synthetic, which can be anionic, cationic, or nonionic. Among these, anionic detergents are used with higher frequency. Examples of anionic detergents include sodium lauryl sulfate and different alkyl benzene sulfonates and alkyl sulfonates. Each molecule has a hydrophobic or lipophilic (nonpolar) segment and a hydrophilic or lipophobic (polar) segment. The ability of a detergent to remove dirt from a surface is attributed to the hydrophobic segment of a molecule. They dissolve the lipid materials of the soil on the surface by forming micelles with the polar segments protruding outside in the water. The concentration of a detergent at which micelle formation starts is called the critical micelle concentration (CMC), which varies with the detergent. The concentration of a detergent is used above its CMC level. Generally, this is ca. 800–900 ppm, but could be 1000–3000 ppm if skin contact does not occur (as in the clean-in-place or CIP method) or where a heavy-duty cleaning is required.

The frequency of cleaning depends on the products being processed and the commitment of the management to good sanitation. From a microbiological standpoint, prior microbiological evaluation of a product can give an indication about the frequency of cleaning necessary in a particular facility. Cleaning of the equipment is done either after disassembling the equipment or by the CIP system. Because of its efficiency and lower cost, CIP cleaning has become popular. The system uses detergent solutions at a high pressure. Because microorganisms can grow in some detergent solutions, they preferably should be prepared fresh (not exceeding 48 h).[1–4]

G. Sanitation of Food-Processing Equipment

Efficient cleaning can remove some microorganisms along with the soil from the food contact surfaces, but cannot ensure complete removal of pathogens. To achieve this goal, food contact surfaces are subjected to sanitation after cleaning. The methods should effectively destroy pathogenic microorganisms as well as reduce total microbial load. Several physical and chemical methods are used for sanitation of food-processing equipment.

Physical agents used for sanitation of food-processing equipment include hot water, steam, hot air, and UV irradiation. UV irradiation is used to disinfect surfaces and is discussed in Chapter 38. Hot water and steam, although less costly and efficient for destroying vegetative cells, viruses, and spores (especially steam) can be used only in a limited way.

Chemical sanitizers are used more frequently than physical sanitizers. Several groups of sanitizers are approved for use in food-processing plants. They vary greatly in their antimicrobial efficiency. Some of the desirable characteristics used in selecting a chemical sanitizer are effectiveness for a specific need, nontoxicity, noncorrosiveness, no effect on food quality, easy to use and rinse, stability, and cost effectiveness. Important factors for antimicrobial efficiency are exposure time, temperature, concentrations used, pH, microbial load and type, microbial attachment to surface, and water hardness. Microbial attachment has been discussed separately (Appendix A). The mechanisms of antimicrobial action and the advantages and disadvantages of some of the sanitizers used in food-processing plants are briefly discussed here. Some sanitizers, designated as detergent sanitizers, can both clean and sanitize. They can be used in a single operation instead of first using detergent to remove the soil and then using sanitizers to control microorganisms. They are also discussed here.[1–4]

1. Chlorine-Based Sanitizers

Some of the chlorine compounds used as sanitizers are liquid chlorine, hypochlorites, inorganic or organic chloramines, and chlorine dioxide. Chlorine compounds are effective against vegetative cells of bacteria, yeasts and molds, spores, and viruses. Clostridial spores are more sensitive to chlorine compounds than are bacilli spores. The antimicrobial (germicidal) action of chlorine compounds is due to the oxidizing effect of chlorine on the –SH group in many enzymes and structural proteins. In

addition, damage to membrane, disruption of protein synthesis, reactions with nucleic acids, and interference with metabolisms have been suggested.

The germicidal action of liquid chlorine and hypochlorites is produced by hypochlorous acid (HOCl). It probably enters the cell and reacts with the –SH group of proteins. HOCl is stable at acid pH and is thus more effective; at alkaline pH, it dissociates to H^+ and OCl^- (hypochlorite ions), which reduces its germicidal effectiveness. They are also less effective in the presence of organic matter. Chloramines (inorganic or organic), such as Chloramine T, release chlorine slowly, but they are less active against bacterial spores and viruses. They are effective, to some extent, against vegetative cells at alkaline pH. Chlorine dioxide is more effective at alkaline pH and in the presence of organic matter.

Chlorine compounds are fast acting against all types of microorganisms, less costly, and easy to use. However, they are unstable (especially at higher temperatures and with organic matter), corrosive to metals, can oxidize food (color, lipid), and are less active in hard water.[1–4]

2. Iodophores

Iodophores are prepared by combining iodine with surface-active compounds, such as alkylphenoxypolyglycol. Because of the surface-active compounds, they are relatively soluble in water. Iodophores are effective against Gram-positive and Gram-negative bacteria, bacterial spores, viruses, and fungi. Their germicidal property is attributed to elemental iodine (I_2) and hypoiodous acid, which oxidize the –SH group of proteins, including key enzymes. They are more effective at acidic pH and higher temperatures. In the presence of organic matters, they do not lose germicidal property as rapidly as chlorine does. However, their effectiveness is reduced in hard water.

They are fast acting, noncorrosive, easy to use, nonirritating, and stable. However, they are expensive, less effective than hypochlorites against spores and viruses, can cause flavor problems in products, and react with starch.[1–4]

3. Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) can be used as detergent sanitizers because they have cleaning properties along with germicidal abilities. However, they are principally used as sanitizers. They are synthesized by reacting tertiary amines with alkyl halides or benzyl chloride. The general structure is:

$$\left[\begin{array}{ccccc} & & R_2 & + & \\ & & \cdot\cdot & & \\ R_1 & : & N & : & R_3 \\ & & \cdot\cdot & & \\ & & R_4 & & \end{array}\right] Cl^- \text{ or } Br^-$$

where R_1, R_2, R_3, and R_4 represent alkyl and other groups. The cationic group is hydrophobic and the anionic group is hydrophilic. QACs can act as bactericides in high concentrations and when used in solution. However, they form a film on the equipment surface, in which state (low concentrations) they are bacteriostatic. They are more effective against Gram-positive bacteria than many Gram-negative bacteria, bacterial spores, fungi, and viruses. The antimicrobial action is produced by the denaturation of microbial proteins and destabilization of membrane functions. They are more effective against microorganisms at acidic pH and higher temperature. Their effectiveness is not greatly reduced in the presence of organic matters. However, they are less effective in hard water.

QACs are advantageous as sanitizers because they are highly stable, noncorrosive, nonirritating, nontoxic, show residual bacteriostatic effect (however, they need to be rinsed before use of the equipment), and show detergent effect. The disadvantages are high cost; low activity against many Gram-negatives, spores, and viruses; incompatibility with anionic synthetic detergents; and rinsing requirement before use because of film formation on equipment surfaces. Some Gram-negative bacteria, such as *Pseudomonas* spp., can grow in diluted QAC solutions.[1–4]

4. H_2O_2

H_2O_2 is a very effective germicide and kills vegetative cells, spores, and viruses. The use of H_2O_2 solutions in food (milk and liquid egg) is discussed in Chapter 16 and Chapter 37. Its use as a sanitizer is briefly described here. The U.S. Food and Drug Administration has approved its use for sanitation of equipment and containers used in the aseptic packaging of foods and beverages. Equipment and container surfaces can be sterilized in 15 min with a 30 to 50% solution; the treatment time can be reduced if the temperature of the solution is raised to 150 to 160ºF (65.6 to 71.7°C). Use of H_2O_2 in vapor phase can also be effective in killing microorganisms on food contact surfaces. Organic materials greatly reduce the germicidal effect of H_2O_2.[1–4]

H. Microbiological Standards, Specifications, and Guidelines

Microbiological standards, specifications, and guidelines are useful in keeping the microbial load of foods at acceptable levels by various methods, one of which is by controlling their access to foods. Microbiological standards of food are set and enforced by regulatory agencies to increase consumer safety and product stability. A standard dictates the maximum microbial level that can be accepted in a food. With proper sanitation and quality control, this level is generally attainable. Some examples are maximum acceptable levels of standard plate counts (SPCs) of Grade A raw milk, 100,000/ml; pasteurized Grade A milk, SPC 20,000/ml and coliforms ≤10/ml. However, very few foods have microbiological standards. Instead, many foods and food ingredients have microbiological specifications. A specification indicates maximum permissible microbial load for the acceptance of a food or food ingredient. It should be attainable and agreed on by the buyers and sellers of the products. It is not set up or enforced by regulatory agencies. In the U.S., the military

has microbiological specifications of foods purchased outside for army rations. For example, dried whole egg has the following specifications: aerobic plate count (APCs), 25,000/g; coliforms, 10/g; and *Salmonella*, negative in 25 g. The specifications discourage mixing of a microbiologically poor-quality product with a good-quality product. Microbiological guidelines are generally set either by regulatory agencies or food processors to help generate products of acceptable microbiological qualities. A guideline is set at a level that can be achieved if a food-processing facility uses good cleaning, sanitation, and handling procedures. It also helps detect if a failure has occurred during processing and handling, and thus alerts the processor to take corrective measures.[1–4]

IV. CONCLUSION

Spoilage and pathogenic microorganisms enter in food from different sources. One of the major objectives to produce a safe food with desirable shelf life is to minimize the access of microorganisms in food from various sources. This can be achieved by proper plant design, training personnel, designing equipment that can be sanitized effectively, and establishing an efficient cleaning and sanitation procedure. Many cleaning and sanitizing chemicals are available commercially. The aim will be to select agents that are suitable for a specific purpose. Adaptation of an efficient and approved procedure (by regulatory agencies) helps meet the required microbiological standards and specifications. Chapter 31 describes methods used to remove microorganisms from food to keep their initial load at a low level.

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QUESTIONS

1. Discuss the objectives of food sanitation.
2. List five factors that are important in reducing microbial access to foods.
3. Describe how water, ice, brine, and curing solution can contribute to the microbial load in a food.
4. List the objectives of using cleaning agents in food-processing facilities. Describe the properties of a desirable detergent for use in a food-processing plant.

5. Discuss the functions of sanitizers for use in food. Describe the advantages and disadvantages of the following agents as sanitizers: hot water, steam, UV light, hypochlorite, iodophores, QACs, and H_2O_2.
6. Define microbiological standards, microbiological specifications, and microbiological guidelines. Explain briefly how they help to keep microbial load in a food at an acceptable level.

CHAPTER 31

Control by Physical Removal

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I. INTRODUCTION

Microorganisms can be physically removed from solid and liquid foods by several methods. In general, these methods can partially remove microorganisms from food, and by doing so they reduce the microbial level and help other antimicrobial steps that follow to become more effective. They are generally used with raw foods before further processing.

II. PHYSICAL METHODS

A. Centrifugation

Centrifugation[1] is used in some liquid foods, such as milk, fruit juices, and syrups, to remove suspended undesirable particles (dust, leucocytes, and food particles). The process consists of exposing the food in thin layers to a high centrifugal force.

The heavier particles move outward and are separated from the lighter liquid mass. Although this is not intended to remove microorganisms, spores, large bacterial rods, bacterial clamps and chains, yeasts and molds can be removed because of their heavier mass. Under high force, as much as 90% of the microbial population can be removed. Following centrifugation, a food will have fewer thermoduric microorganisms (bacterial spores) that otherwise would have survived mild heat treatment (e.g., milk pasteurization) and increased the microbial load of the pasteurized product.

B. Filtration

Filtration[2] is used in some liquid foods, such as soft drinks, fruit juices, beer, wine, and water, to remove undesirable solids and microorganisms and to give a sparkling clear appearance. As heating is avoided or given only at minimum levels, the natural flavors of the products and heat-sensitive nutrients (e.g., vitamin C in citrus juices) are retained to give the products natural characteristics. The filtration process can also be used as a step in the production of concentrated juice with better flavor and higher vitamins. Many types of filtration systems are available. In many filtration processes, coarse filters are used initially to remove large components; this is followed by ultrafiltration. Ultrafiltration methods, depending on pore size of the filter materials (0.45 to 0.7 μm), are effective in removing yeasts, molds, and most bacterial cells and spores from liquid products.

Filtration of air is also used in some food-processing operations, such as spray drying of milk, to remove dust from air used for drying. The process also removes some microorganisms with dust, and this reduces the microbial level in food from air.[2]

C. Trimming

Fruits and vegetables showing damage (greater chance of microbial contamination) and spoilage are generally trimmed.[2] In this manner, areas heavily contaminated with microorganisms are removed. Trimming the outside leaves in cabbage used for sauerkraut production also helps reduce microorganisms coming from soil. Trimming is also practiced for the same reason to remove visible mold growth from hard cheeses, fermented sausages, bread, and some low-pH products. However, if a mold strain is a mycotoxin producer, trimming does not ensure removal of toxins from the remaining food. Trimming is also used regularly to remove fecal stain marks, unusual growths, and abscesses or small, infected areas from carcasses of food animals and birds. Although this method helps remove highly contaminated areas, it does not ensure complete removal of the causative microorganisms. Thus, a beef carcass can have an area contaminated with feces along with enteric pathogens. Merely removing the visibly tainted area by trimming does not help remove pathogens from the surrounding areas that do not show the taint. This is an important concern in the production of safer foods.

D. Washing

Washing equipment and work areas has been discussed under cleaning and sanitation in Chapter 30. Here, only washing foods is discussed.[3–6] Fruits and vegetables are washed regularly to reduce temperature (which helps reduce the metabolic rate of a produce and microbial growth) and remove soil. Washing also helps remove the microorganisms present, especially from the soil. It is also used for shell eggs to remove fecal materials and dirt. During the processing of chicken and turkey, the carcasses are exposed to water several times. During defeathering, they are exposed to hot water; following removal of the gut materials, they are given spray washings; and finally they are exposed to cold water in a chilling tank. Although these treatments are expected to reduce microbial load, they can spread contamination of undesirable microorganisms, particularly enteric pathogens. Thus, higher percentages of chicken have been demonstrated to be contaminated with salmonellae when coming out of the chill tank than before entering the tank. This aspect is discussed in Chapter 35. Carcasses of food animals, such as beef, pork, and lamb, are washed to remove hair, soil particles, and microorganisms. Instead of hand washing, automated machine-washing at a high pressure is currently used to effectively remove undesirable materials and microorganisms from the carcass. In addition to high pressure, the effectiveness of hot water, steam, ozonated water, and water containing chlorine, acetic acid, propionic acid, lactic acid, tripolyphosphates, or bacteriocins (nisin and pediocin) of lactic acid bacteria has been studied for removing microorganisms, particularly enteric pathogens such as *Salmonella* spp., *Campylobacter jejuni, Escherichia coli* O157:H7, and *Listeria monocytogenes*, separately or in combinations. The results were not consistent. Some of these agents also have a bactericidal property. However, studies show that all the agents can reduce bacterial contamination to a certain level from the carcass surfaces, and a combination of two or more components may be better.

The suitability of the combinations as well as their concentrations and duration of application must be determined. One has to recognize that, with time, microorganisms can form a biofilm on the carcass surface (Appendix A). The nature of the biofilm varies with microbial species and strains. Also, with time, the biofilm becomes more stable, and removing microorganisms by washing after the formation of a stable biofilm is relatively difficult. This aspect needs to be considered while developing effective methods of carcass washing. Removal of pathogens from carcass surfaces will be an important area to develop a suitable intervention strategy for ensuring the safety of meat and meat products (see Appendix D).

III. CONCLUSION

Microorganisms that have gained access to food can be controlled by various means, one of which is to remove them physically. This can be achieved by the different methods discussed in this chapter. Some of them are used in liquid foods, but have limited application; others are used in solid foods. Chapter 32 discusses different methods of heating to kill microorganisms already present in food.

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QUESTIONS

1. List the methods used to remove microorganisms from foods and discuss one advantage and one disadvantage of each method.
2. Discuss two specific microbiological disadvantages, with examples, of trimming food for visible microbial growth or microbial contamination.
3. Discuss two specific microbiological disadvantages, with examples, of washing carcasses of animals and birds to reduce microorganisms.
4. List six different agents that have been tested, and discuss their relative efficiency in removing microorganisms from beef carcasses.
5. A milk processor found that a batch of milk had very high levels of bacterial spores that could increase the SPC level following pasteurization. Discuss a physical removal procedure that he could use to remove the spores to attain the desired SPC level.

CHAPTER 32

Control by Heat

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I. INTRODUCTION

The desirable effect of heat (from fire) on the taste of foods of animal and plant origin, especially seeds, tubers, and roots, was probably accidentally discovered, following a natural forest fire, by our ancestors long before civilization. They also possibly recognized that heated foods did not spoil as rapidly as raw foods or cause health hazards. Since then, particularly following the invention of pottery and ovens, heat has been used to roast, boil, bake, and concentrate foods to improve taste and to enhance shelf life (and probably safety). However, it was not until ca. 1810 that

extended shelf life of perishable foods was achieved by a specific method, appertization. Nicolas Appert, after whom the method was named in France, reported that by filling a clean glass jar with a desirable food, heating the contents in boiling water for 6 h or more, and hermetically sealing the container with a cork kept the food unspoiled and safe for a long time. Some of his products (meat stew) prepared in 1824 were found nontoxic when opened in 1938, but were found to contain dormant spores. Heating of foods in cans was developed by Durand in England in 1810, and by 1820 the method was used in the U.S. Methods to reduce the heating time to ca. 30 min were also developed by adding different types of salts in water to raise the boiling point above 212ºF (100ºC). In 1870, the autoclave was invented, providing the possibility of developing pressure vessels that could be used to heat canned food at a much higher temperature for a relatively shorter period of time in order to retain the quality of foods as well as to enhance shelf life.

All these developments occurred without clearly knowing why a perishable food, following appertization or canning, did not spoil (or cause foodborne disease). The role of microorganisms in the spoilage of wine and milk was discovered by Louis Pasteur ca. 1870. He also showed that mild heat treatment (pasteurization) killed these microorganisms, and in the absence of recontamination, the products stayed unspoiled. Subsequently, the microbial role in foodborne diseases was also recognized. Following these, studies were conducted to isolate the most heat-resistant microorganisms (spores) that could survive heating in canned foods and cause spoilage and foodborne diseases and to determine the temperature and time requirements for their destruction. Studies were also conducted to identify the time–temperature relationships for the destruction of less heat-resistant microorganisms (vegetative cells). From these results, mathematical expressions were developed to accurately predict time–temperature relationships to destroy microorganisms (also some enzymes and toxins) by heating foods at different temperatures and times.

II. OBJECTIVES

The main objective (microbiological) of heating food is to destroy vegetative cells and spores of microorganisms that include molds, yeasts, bacteria, and viruses (including bacteriophages).[1] Although very drastic heat treatment (sterilization) can be used to kill all the microorganisms present in a food, most foods are heated to destroy specific pathogens and some spoilage microorganisms, which are important in a food. This is necessary in order to retain the acceptance and nutritional qualities of a food. To control growth of surviving microorganisms in the food, other control methods are used following heat treatment.

Heating of foods also helps destroy undesirable enzymes (microbial and food) that would otherwise adversely affect the acceptance quality of food. Some microorganisms also produce heat-stable proteinases and lipases in food. Heating a food to a desired temperature for a specific time can help destroy or reduce the activity of these enzymes. This is especially important in foods stored for a long time at room temperature.

Some microorganisms can release toxins in food; also, some foods can have natural toxins. If a toxin is heat sensitive, sufficient heating will destroy it and consumption of such a food will not cause health hazards. It is also important to recognize that microbial (and natural) heat-stable toxins are not completely destroyed even after high heat treatment.

Heating (warming) of ready-to-eat foods before serving is also usually used to prevent growth of pathogenic and spoilage microorganisms. A temperature above 50ºC, preferably 60ºC, is important to control growth of many microorganisms in such foods during storage before serving.

Finally, heating of raw materials, such as milk, is done before adding starter culture bacteria for fermentation to kill undesired microorganisms (including bacteriophages) and to allow growth of the starter cultures without competition.

III. MECHANISM OF ANTIMICROBIAL ACTION

Depending on the temperature and time of heating, microbial cells and spores can be heat-shocked, sublethally injured, or dead. Heat-shocked cells acquire some resistance to subsequent heating and sublethally injured cells and spores retain the ability to repair and multiply. These are discussed in Chapter 9.

Results of different studies have shown that following heat injury, bacterial cells show loss of permeability and increased sensitivity to some compounds to which they are normally resistant. Sublethally injured cells seem to suffer injury in the cell membrane, cell wall, DNA (strand break), ribosomal RNA (degradation), and some important enzymes (denaturation). Death occurs from damages in some vital functional and structural components. Bacterial spores, following heating, were found to lose structural components from the spore coat, suffer damage to the structures that are destined to become membrane and wall, and develop an inability to use water for hydration during germination. Death results from the inability of a spore either to germinate or to outgrow (see Chapter 9).

Exposure of microbial cells to ca. 45 to 50ºC for a short time, which can occur while heating a large volume of a food such as a large rare roast, may induce production of heat shock proteins (stress proteins) by the cells. In the presence of these proteins, the microbial cells can develop relatively greater resistance to subsequent heating at higher temperatures (see Chapter 9). The implication of this phenomenon in the thermal destruction of microbial cells in low-heat-processed foods is important.[2]

IV. INFLUENCING FACTORS

The effectiveness of heat in killing microbial cells and spores depends on many factors, some of which are related to the inherent nature of the foods, and others to both the nature of microorganisms and the nature of processing. An understanding of these factors is important to develop and adopt an effective heat-processing procedure for a food.[1]

A. Nature of Food

Composition (amount of carbohydrates, proteins, lipids, and solutes), A_w (moisture), pH, and antimicrobial content (natural or added) greatly influence microbial destruction by heat in a food. In general, carbohydrates, proteins, lipids, and solutes provide protection to microorganisms against heat. Greater microbial resistance results with higher concentrations of these components. Microorganisms in liquid food and food containing small-sized particles suspended in a liquid are more susceptible to heat destruction than in a solid food or a food with large chunks in liquid. Microorganisms are more susceptible to heat damage in a food that has higher A_w or lower pH. In low-pH foods, heating is more lethal to microorganisms in the presence of acetic, propionic, and lactic acids than phosphoric or citric acid at the same pH. In the presence of antimicrobials, not inactivated by heat, microorganisms are destroyed more rapidly, the rate differing with the nature of the antimicrobials.

B. Nature of Microorganisms

Factors that influence microbial sensitivity to heat include inherent resistance of species and strains, stage of growth, previous exposure to heat, and initial load. In general, vegetative cells of molds, yeasts, and bacteria are more sensitive than spores. Cells of molds, yeasts, and many bacteria (except thermoduric and thermophilic), as well as viruses, are destroyed within 10 min at 65ºC. Most thermoduric and thermophilic bacterial cells important in foods are destroyed in 5 to 10 min at 75 to 80ºC. Yeast and most mold spores are destroyed at 65 to 70ºC in a few minutes, but spores of some molds can survive at as high as 90ºC for 4 to 5 h. Bacterial spores vary greatly in their sensitivity to heat. Generally, heating at 80 to 85ºC for a few minutes does not kill them. Many are destroyed at 100ºC in 30 min, but there are bacterial species whose spores are not destroyed even after boiling (100ºC) for 24 h. All spores are destroyed at 121ºC in 15 min (sterilization temperature and time). Below this temperature (and time), spores of some bacterial species can survive; however, this depends on the initial number of spores and the nature of the suspending medium. Species and strains of bacterial cells and spores also differ in heat sensitivity. This is especially important if a food is heat treated on the basis of results obtained by using a heat-sensitive species or strain but contains heat-resistant variants.

Cells at the exponential stage of growth are more susceptible to heat than resting cells (stationary phase). Also, cells previously exposed to low heat become relatively resistant to subsequent heat treatment (due to synthesis of stress proteins). Finally, the higher the initial microbial load in a food, the longer the time it takes at a given temperature to reduce the population to a predetermined level. This is because the rate of heat destruction of microorganisms follows first-order kinetics, which is discussed later. This suggests the importance of lower initial microbial loads (through sanitation and controlling growth) in a food before heat treatment.

C. Nature of Process

Microbial destruction in food by heat is expressed in terms of its exposure to a specific temperature for a period of time, and these are inversely related: the higher the temperature, the shorter the period of time required to get the same amount of destruction when other factors are kept constant. As a food is heated by conduction (molecule-to-molecule energy transfer) and convection (movement of heated molecules), a liquid food is heated more rapidly than a solid food, and a container with high conduction (metal) is better. Also, food in a small container is heated more rapidly than in a large container. A product can have a cold point at the center (in a solid food in a can) or near the end (in a liquid food in a can), which may not attain the desired temperature within the given time. Finally, it needs to be emphasized that heating a food at a given temperature for a specific time means that every particle of that food should be heated to the specified temperature (say 71.6ºC or 161ºF) and stay at that temperature for the specified time (15 sec; used in milk pasteurization). This time is also called the holding time. The time during which milk is heated before the temperature reaches 161ºF in this case is not considered (but considered in the commercial sterilization process, which uses a much higher temperature).

V. MATHEMATICAL EXPRESSIONS

When a population of microbial cell suspension is heated at a specific temperature, the cells die at a constant rate. This observation helps in expressing the microbial death rate due to heat as a function of time and temperature under a given condition. These expressions are helpful to design a heat-treatment method for a food.

A. Decimal Reduction Time (*D* Value)

The *D* value is the time in minutes during which the number of a specific microbial (cells or spores) population exposed to a specific temperature is reduced by 90% or 1 log. It is expressed as $D_T = t$ min, where T is the temperature (ºC or ºF) and t is the time (min) for 1 log reduction of the microbial strain used. Thus, it is a measure of heat sensitivity of microorganisms and varies with microbial species and strains, temperature used, and other variables, such as suspending media and age of the culture.

It can be determined by using the expression:

$$D_T = \frac{t}{\log_{10} x - \log_{10} y}$$

where x and y represent, respectively, microbial numbers before and after exposing at temperature T for t min. It also can be determined by plotting $\log_{10}$ survivors

against time of exposure (min) for a specific temperature (Figure 32.1). Ideally, it is a straight-line graph and is independent of the initial number of a microbial population. It can be extrapolated to $-\log_{10}$ values to obtain very low levels of microbial survivors, such as 1 cell or spore in 10 g, or 100 g, or 1000 g of a product, and thus can be used to design heat treatment parameters to obtain a desirable low level of a microbial population in a food. It is evident from the plot that to reach a desirable microbial level, a food with lower initial numbers will require less time (fewer *D*) than a food with higher initial numbers at a specific temperature. It can also be used to determine the relative sensitivity of two or more microbial species or strains with respect to a specific temperature (Figure 32.1).

The 12*D* concept is used in heat processing of high-pH foods (pH > 4.6, low-acid foods such as corn, beans, and meat) to destroy the most heat-resistant spores of the pathogenic bacteria *Clostridium botulinum*. It means that the products are given heat treatment to reduce the population of *Clo. botulinum* spores by 12 log cycles. Hypothetically, if 1 billion (10^9) cans at one time, each containing 10^3 spores of *Clo. botulinum*, are given proper heat treatment, only 1 can will contain 1 viable spore. This is an extreme processing condition used for a high degree of safety. The 12*D* value at $D_{121.1^{\circ}C}$ is ca. 2.8 or ca. 3.0 min.[1,3]

B. Thermal Death Time (TDT), *Z* Value, and *F* Value

TDT is the time in log that is necessary to completely destroy a specific number of microbial cells or spores in a population at a specific temperature. It indicates the relative sensitivity of a microorganism to different temperatures. A TDT curve can be constructed either by plotting log time of complete destruction against temperature or by plotting log *D* values against temperature (Figure 32.2; this is called a phantom TDT curve). The slope of the curve is the *Z* value, which indicates the temperature (ºC or ºF) required to change the *D* value (or TDT) to transverse by 1 log. A value of *Z* = 10 in ºC implies that if the *D* value of bacterial spores at 100ºC is 50 min, at 110ºC it will be 5 min, and at 120ºC it will be 0.5 min. In developing heat-processing conditions of a food, *D* and *Z* values are used to obtain desirable destruction of microorganisms (cells and spores). In addition, the symbol *F* is used to express the time (min) necessary to completely destroy a specific number of microbial spores or cells at a reference temperature (121.1ºC for spores and 60ºC for cells). $F_{121.1^{\circ}C}$ for *Clo. botulinum* Type A spores was found to be 0.23 min and was used to calculate the 12*D* value ($12 \times 0.23 = 2.78$ or 3 min) for heat processing of low-acid canned foods.

VI. METHODS

On the basis of temperature and time of heating the food used to destroy microorganisms, the methods can be broadly divided as low-heat processing and high-heat processing. Low-heat processing is used to destroy mainly the microorganisms relatively sensitive to heat and is not generally effective against thermoduric micro-

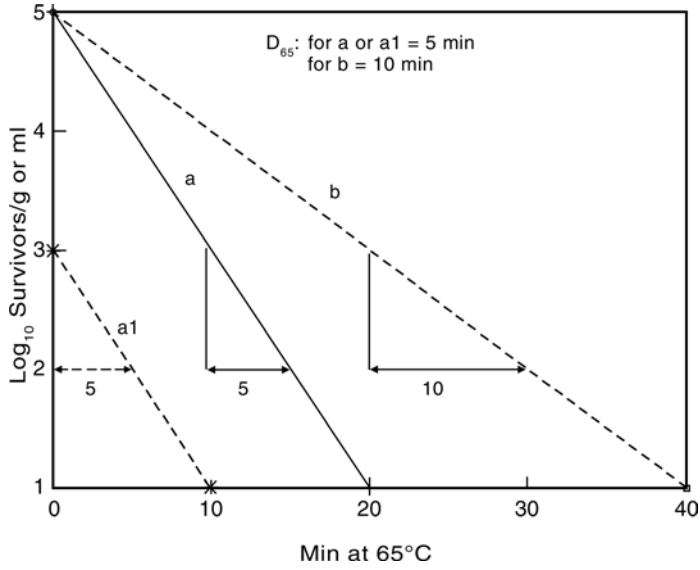


Figure 32.1 Graphical presentation of decimal reduction time (*D*). The graph also illustrates the number of *D* required with high and low populations of bacteria with the same heat sensitivity (a and a1) to obtain a desired survivor level (say 10^1/g or /ml) and different *D* values for two bacterial species with different heat sensitivities at 65ºC (a and b).

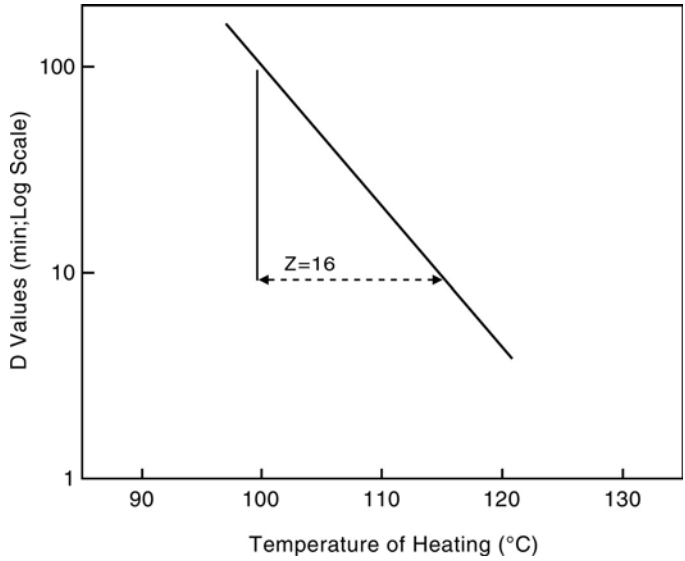


Figure 32.2 Hypothetical thermal death time curve. *D*, decimal reduction time; *Z*, ºC required for the thermal death time curve to transverse over a log_{10} cycle.

organisms. In contrast, high-heat processing is used to destroy thermodurics, and especially the most heat-resistant spores of spoilage and pathogenic bacteria. Methods (e.g., baking, broiling, simmering, roasting, and frying) used in cooking foods and blanching used to destroy some natural enzymes in fresh vegetables and fruits are not discussed here. Although they destroy microorganisms, their main use is for food preparation and not destruction of microorganisms in foods.[1,4]

A. Low-Heat Processing or Pasteurization

The temperature used for low-heat processing or pasteurization is below 100ºC. The objectives of pasteurization are to destroy all the vegetative cells of the pathogens and a large number (~90%) of associative (many of which can cause spoilage) microorganisms (yeasts, molds, bacteria, and viruses). In certain foods, pasteurization also destroys some natural enzymes (e.g., phophatase in milk). The temperature and time are set to the lowest level to meet the microbiological objectives and to minimize thermal damage of the food, which otherwise could reduce the acceptance quality (such as heated flavor in milk) or pose processing difficulties (such as coagulation of liquid egg). Depending on the temperature used, thermoduric cells of spoilage bacteria and spores of pathogenic and spoilage bacteria survive the treatment. Thus, additional methods need to be used to control the growth of survivors (as well as postpasteurization contaminants) of pasteurized products, unless a product has a natural safety factor (e.g., low pH in some acid products). Refrigeration, modified atmosphere packaging, incorporation of preservatives, reduction of $A_{и}$, and other techniques are used, when possible in combination, to prevent or retard the problem of microbial growth in low-heat-processed products. Microbial heat-stable enzymes and toxins are not destroyed unless a food is heated for 30 min or longer at or above 90ºC.

Pasteurization of milk has been used for a long time. Two methods, heating at 145ºF (62.8ºC) for 30 min or 161ºF (71.7ºC) for 15 sec, are used to destroy the most heat resistant Q fever pathogen, *Coxiella burnetii*. The methods are also designated, respectively, as low temperature long time (LTLT) and high temperature short time (HTST) methods. As indicated before, regulation requires every particle of milk to be heated at 145ºF for 30 min or at 161ºF for 15 sec (holding time). Foods that are not uniformly heated to the specified temperature and time can be involved in foodborne diseases. Immediately after the holding time, the milk is cooled to 40ºF, packaged, and maintained at that temperature until consumed. Low-heat treatment is used for processing many products by using different times and temperatures, e.g., ice cream mix, 180ºF (82.3ºC) for 25 sec or 160ºF (71.2ºC) for 30 min; liquid whole egg, 140ºF (60ºC) for 3.5 min (intended to destroy *Salmonella*); fruit juices, 60 to 70ºC for 15 min or 80 to 85ºC for 1 min; wine, 82 to 85ºC for 1 min; pickles in acid (pH 3.7), 74ºC for 15 min; vinegar, 65.6 to 71.1ºC for 1 min or 60ºC for 30 min (heating of all low-pH products is designed to destroy aciduric spoilage microorganisms); crab meat, 70ºC for 10 min; low-heat-processed meat products, 60 to 70ºC internal temperature (depending on the size, it can take 2 h for the center of a product to reach the desired temperature; heating is not intended to kill *Clo.*

botulinum Type A or nonproteolytic B spores). The time–temperature of pasteurization for different foods is also specified by regulatory agencies.

In addition to the use of hot water (e.g., for milk) or moist heat (e.g., for meat products) to heat process some foods, other products, such as dried egg whites and dried coconut, are pasteurized by dry heat. In such a condition, the products are exposed to 50 to 70ºC for 5 to 7 d.

In the production of some fermented foods, the raw materials are heated to a high temperature to destroy vegetative cells of pathogens and spoilage microorganisms, which include thermoduric bacterial cells. Raw milk used for the production of buttermilk, acidophilus milk, and yogurt are given a 30- to 60-min heat treatment at ca. 90ºC before adding starter-culture bacteria. Heating helps the starter culture bacteria grow preferentially, in addition to improving the gelling properties of the milk proteins at low pH.[1,4]

B. High-Heat-Processed Foods

The process involves heating foods at or above 100ºC for a desired period of time. The temperature and time of heating are selected on the basis of product characteristics and the specific microorganisms to be destroyed. Most products are given a commercially sterile treatment to destroy microorganisms growing in a product under normal storage conditions. Low-acid or high-pH (pH > 4.6) products are given 12*D* treatment to destroy *Clo. botulinum* Type A and B spores (the most resistant spores of a pathogen). However, the products can have viable spores of thermophilic spoilage bacteria (e.g., *Bacillus stearothermophilus, Bac. coagulans, Clo. thermosaccharolyticum,* and *Desulfotomaculum nigrificans*; see Chapter 19). As long as the products are stored at or below 30ºC, these spores will not germinate. If the products are temperature abused to 40ºC and above even for a short time, the spores will germinate. Subsequent storage at or below 30ºC will not prevent outgrowth and multiplication of these thermophiles to cause food spoilage. The time and temperature required for commercial sterility of a particular food are determined by actual pack inoculation studies. Generally, *Clo. sporogenes* PA 3679 is used to simulate *Clo. botulinum* because this is a nonpathogenic strain, but the spores have the same heat resistance as *Clo. botulinum* Type A or B (both proteolytic and nonproteolytic). For spoilage control studies, spores of *Bac. stearothermophilus* are used because spores of this species are most heat resistant.

For high-acid or low-pH (pH ≤ 4.6) products, such as tomato products, fruit products, and acidified foods, a much lower heat treatment is used. Because *Clo. botulinum* spores cannot germinate or grow at this low pH, their presence is of little health hazard significance. The sporeformers that can germinate and grow in low-pH products (e.g., *Bac. coagulans*) and the aciduric nonsporeforming bacteria (e.g., *Lactobacillus* and *Leuconostoc* spp.), yeasts, and molds that can grow at low pH are relatively heat sensitive. These products are generally heated ca. 100°C for a desirable period of time. High-heat-treated products are either first packed in containers and then heated, or heated first and then packed in sterile containers while still hot (hot pack).

Commercial sterility is also obtained by heating a food at very high temperatures for a short time. This process is designated as ultrahigh temperature (UHT) processing. Milk heated to ca. 150°C for 2 to 3 s can be stored at room temperature (≤30°C) and the products generally have a 3-month shelf life. However, if microbial heat-stable proteinases or lipases are present in the raw milk, the product can show spoilage. In the UHT process, the milk is heated by injecting steam at high pressure for a rapid temperature increase. Following heat treatment in bulk, the milk is packed in small serving containers. Microbial heat-sensitive toxins will be destroyed, but heat-stable toxins may remain active even after heating for commercial sterility.[1,4]

Under special circumstances, foods are heated to destroy all microorganisms (cells and spores) and to achieve sterility. Sterile foods are necessary for immunosuppressant individuals in order to avoid any complications from the microorganisms that are normally present in heated but nonsterile foods.

C. Microwave Heating

Heating or cooking foods by the microwave at home is quite common in both developed and developing countries.[5,6] Frozen foods can be thawed and heated very rapidly, in a few minutes, depending on the size of a product. However, the method has not been well accepted as a source of rapidly generated high heat for commercial operations.

In a microwave oven, the waves change their polarity very quickly. Oppositely charged water molecules in a food rapidly move to align along the waves. The movement of the water molecules generates frictional heat, causing the temperature of the food to rise very rapidly. Depending on the exposure time and intensity of the wave, the temperature can be very high. Microwave treatment is quite lethal to microorganisms and the destruction is caused by the high temperature. At present, microwave-heated foods cannot be considered safe from pathogens. Generally, when a food is heated in a microwave oven, it is not heated uniformly and some areas can remain cold. If a food harbors pathogens, there are chances that they will survive in the cold spots.

VII. CONCLUSION

Heating has been used in food preparation and preservation long before civilization. Following the recognition of microbial roles in food spoilage and foodborne diseases, precise heating techniques have been developed to destroy vegetative cells of yeasts, molds, bacteria, and viruses and spores of bacteria. These include pasteurization, commercial sterilization, and UHT. Destruction of microorganisms by these methods is achieved by heat treatment of a food at a specific temperature for a time period. Microbial destruction at high temperature results from structural and functional destabilization of cells and spores. At a lower heating temperature, the cells and spores can be sublethally injured and stressed. Because they are not dead, these two phenomena have important implications in developing heating methods to destroy microorganisms in food. Because extreme heat to kill all microorganisms cannot

always be used, other methods are used mainly to prevent growth of microorganisms as well as to maintain acceptance qualities of food. One such method is low temperature and is discussed in Chapter 33.

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QUESTIONS

1. Discuss the ideological differences in the use of heating in food preservation before and after 1870 A.D.
2. List the microbiologically related objectives of heating a food.
3. Describe the mechanisms of sublethal and lethal heat injury in bacterial cells and spores. How can stress proteins alter this effect in food processed slowly at low heat?
4. List three important factors that one should consider in designing thermal preservation of a food. Discuss the implications of one of the factors.
5. Define *D* value, *Z* value, *F* value, and 12*D* value. Draw hypothetical plots to show how *D* and *Z* values can indicate relative heat resistance of two microbial species.
6. List the objectives of pasteurization of food. How do these objectives differ from those used in commercial sterilization of foods? Use a food system for each method of treatment to justify your explanations.
7. How does microwave heating differ from conventional heating to ensure food safety?
8. The SPC/ml of raw milk from two suppliers is as follows: (A) SPC/ml 95,000 (with thermodurics 15,000/ml) and (B) SPC/ml 65,000 (with thermodurics 25,000/ml). Briefly discuss *D* value differences that a processor has to adopt to meet the legal bacterial counts for pasteurized Grade A milk. Can both be sold as Grade A pasteurized milk? Explain.

CHAPTER 33

Control by Low Temperature

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I. INTRODUCTION

The effectiveness of low temperature, especially freezing, in food preservation was probably recognized by our ancestors at least in the last Ice Age, before 40,000 B.C. Natural freezing and thawing could also have been used to preserve food during the very early stages of civilization, ca. 10,000 to 12,000 B.C. In the colder regions of the world, foods (e.g., meat, and fish) are still preserved in natural ice. Ice was used by the wealthy Romans to reduce the temperature of foods. Until ca. 1800 A.D., ice blocks from frozen lakes were cut, stored, and used to preserve raw foods (e.g., meats, milk, fish, and produce) by lowering the temperature. By 1840, with the help of ammonia-compressed refrigerator units, ice blocks were produced commercially and used to reduce the temperature of food for preservation. In 1880, refrigeration

was used on ships and trains in Europe to transport meat and fish from other countries. Linde, in Germany, developed the first domestic refrigerator ca. 1874 and started commercial production before 1890. The popularity of domestic refrigerators was initially slow in the U.S.; in 1930, 2 to 3% of the households had the units. In the U.S., methods to freeze fruits and vegetables were developed and commercially used around the 1930s. During this time, retail stores also started using cabinets to display frozen foods.

During World War II (1941 to1944), consumer interest in refrigerated and frozen foods increased dramatically, which helped develop the technology necessary for processing, transporting, retailing, and home storage of refrigerated and frozen foods in the U.S. The popularity of refrigerated and frozen foods has increased steadily since then. In the 1960s, 1970s, and early 1980s, frozen food consumption increased sharply, mainly because of the long shelf life. Since the mid-1980s, there has been increased interest in refrigerated and chilled foods, which consumers view as natural and healthy. The major drawback of many refrigerated foods is their relatively short shelf life, ca. 1 to 2 weeks. But in recent years, several technological improvements, such as oxygen-impermeable packaging materials, good vacuum-packaging equipment, innovative packaging systems, low-temperature refrigeration units (≤–1°C), and use of the hurdle concept to preserve food (Chapter 40) have helped increase the shelf life of many refrigerated foods to 60 d, and for some products, more than 90 d. At present, of the total foods consumed, low-temperature preserved foods constitute more than 65%, and the trend shows a steady increase in the future. To suit the taste of consumers, many new products are being developed that are low in fat (caloric), high in fiber, phosphates, and other additives, and have low amounts or no preservatives. To achieve the long shelf life and to make these products safe, extra precautions are being introduced for microbiological control. This has helped many new or emerging pathogenic and spoilage bacteria, in the absence of competition from associated microorganisms, to become predominant (Chapter 28). New designs of processing equipment for high-production efficiency, centralized production of large volumes of products, transportation of products for long distances in regional storage facilities, retailing conditions, and consumers' handling of the products have facilitated these so-called new pathogenic and spoilage bacteria in gaining prominence (Chapter 20). Unless some effective intervention strategies are developed, new pathogenic and spoilage microorganisms will continue to surface in refrigerated foods stored for a long time.[1–3]

II. OBJECTIVES

The main microbiological objective in low-temperature preservation of food is to prevent or reduce growth of microorganisms. Low temperature also reduces or prevents catalytic activity of microbial enzymes, especially heat-stable proteinases and lipases. Germination of spores is also reduced. Low-temperature storage, especially freezing (and thawing), is also lethal to microbial cells, and, under specific conditions, 90% or more of the population can die during low-temperature preservation. However, the death rate of microorganisms at low temperature, as compared

with that at heat treatment, cannot be predicted (as *D* and *Z* values in heating). Also, spores are not killed at low temperature. Thus, foods are not preserved at low temperature in order to kill microbial cells. Freezing is also used to preserve starter cultures for use in food bioprocessing. This has been discussed in Chapter 13.

III. MECHANISMS OF ANTIMICROBIAL ACTION

The metabolic activities, enzymatic reactions, and growth rates of microorganisms are maximum at the optimum growth temperature. As the temperature is lowered, microbial activities associated with growth slow down. Normally, the generation time, within a certain range, is doubled for every 10°C reduction in temperature. Thus, a species dividing every 60 min in a food at 22°C will take 120 min to divide if the temperature is reduced to 12°C. At lower range, generation time can be even higher than double. For example, if the temperature is reduced from 12°C to 2°C and the species can grow at 2°C, the generation time for the species could be more than 240 min. The lag and exponential phases and the germination time (of spores) for some psychrotrophs (mesophilic types) become increasingly longer as the temperature is reduced to ca. 0°C or even to ca. –1°C. At this temperature, nongrowing cells of some mesophiles (nonpsychrotrophic) and thermophiles may be injured and die, especially if they are stored for a long time (weeks) at 2°C or below and the foods have low A_w, low pH, or preservatives. The rate of catalytic activity of some enzymes also decreases as the temperature of an environment is reduced.[4–6]

Water is present in a food as free water and bound (with the hydrated molecules) water. As the temperature in a food system drops to ca. –2°C, free water in the food starts freezing and forming ice crystals (pure water freezes at 0°C, but in a food with solutes it freezes below 0°C). As the temperature drops further and more ice crystals form, the solutes get concentrated in the remaining water, which in turn depresses the freezing point of the water in the solution. The A_w is also reduced. When the temperature is reduced to ca. –20°C, almost all the free water freezes.

As the temperature of a food is reduced below –2°C, free water inside the microbial cells also undergoes similar changes. At a slow rate of freezing, as the water molecules in the food start freezing, water molecules from inside the microbial cells migrate outside, causing dehydration of cells and concentration of solutes and ions inside. When the temperature is reduced further (above –20°C) so that the water in the food has frozen, water inside the cell also freezes. However, before that, microbial cells are exposed to low pH (due to concentration of ions) and low A_w (due to concentration of solutes) inside and outside the cells. This can cause denaturation and destabilization of the structural and functional macromolecules in the microbial cells, whose stability and functions depend on their three-dimensional structures, and can injure the cells. If the freezing is rapid, very small ice crystals form quickly and the cells are not exposed to the solution effect. This is practiced in the freeze preservation of starter cultures and frozen stock cultures.

Microbial cells subjected to freezing and thawing suffer sublethal (repairable) as well as lethal injury. Studies show that different components of the cell wall (or outer membrane) and cell membrane (or inner membrane) are injured. DNA strand

break, ribosomal RNA degradation, and activation and inactivation of some enzymes have also been reported in some studies. In sublethally injured cells, the structural and functional injuries are reversible. In lethally injured (or dead) cells, the damages are irreversible (Chapter 9).[4–7]

IV. INFLUENCING FACTORS

The effectiveness of low temperature in controlling microbial growth and microbial enzymatic activity in food depends on many factors. These factors can be arranged into three groups: those unique to low-temperature, those related to the food environment, and those inherent in microorganisms. These factors not only help prevent or reduce the growth of microorganisms but can also greatly influence the extent of sublethal and lethal injury that microorganisms incur in food preserved at low temperatures. An understanding of the influence of these factors and interaction among them is important to design an efficient and predictable method to preserve a specific food at low temperature.

A. Nature of Process

At temperatures above freezing of free water (≤–2°C), different types of bacteria, molds, and yeasts can grow in a food. But the lag and exponential phases become longer as the temperature is reduced. In the low range, even a difference in <1°C can be highly important. A *Pseudomonas fluorescens* strain was reported to have a generation time of ca. 6.7 h at 0.5°C, but 32.2 h at 0°C. Thus, a reduction in 0.5°C increased the generation time by ca. 4.5-fold. This is much more than the theoretical estimate, which suggests that the generation time doubles for every 10°C reduction. Spores of some spoilage *Bacillus* and *Clostridium* spp. can germinate even at refrigeration temperature (4.5°C or 40°F). Cells of some mesophiles and thermophiles can be sublethally injured as well as die as the temperature drops below 4.5°C.

As the temperature is reduced enough to cause a large portion of the water to freeze, the growth of most microorganisms stops except for some psychrophilic bacteria, yeasts, and molds. Although there are conflicting reports, slow growth probably can occur up to –10°C, especially by some molds. As the temperature drops further, to ca. –20°C, and water in a food freezes completely, more cells will have sublethal and lethal injury. The rate of freezing and the lowest temperature of freezing dictate the extent of microbial damage from ice crystals. Damage and death are more extensive at a slower rate of freezing and at –20ºC than at a rapid rate of freezing and at –78°C or –196°C (temperatures of solid CO_2 and liquid N_2, respectively). Death and sublethal injury are very high during initial storage (ca. 7 d) but subsequently slow down.

Fluctuation of temperature of a food during low-temperature storage has great impact on growth, sublethal injury, and death of microorganisms. This quite readily happens to foods during storage, transport, retail display, and at home. A fluctuation of temperature of food from ≤4.4°C to 10–12°C not only stimulates rapid growth of psychrotrophic pathogenic and spoilage bacteria, but many mesophilic spoilage

and pathogenic bacteria start to grow and their spores germinate at this range. Just from the spoilage aspect, a 6- to 8-h temperature abuse (12°C) of a vacuum-packaged, refrigerated, low-heat-processed meat product can reduce its expected shelf life of 8 weeks by ca. 7 to 10 d. A fluctuation in temperature of a frozen food increases microbial death and injury due to repeated damaging solution effect and mechanical damage from larger ice crystals that form during repeated freezing and thawing. Dead microbial cells can also lyse, releasing intracellular enzymes, many of which (e.g., proteinases and lipases) can act on food components and reduce the acceptance quality of food (see Chapter 21).

The rate of cooling of a food is also very important for effective control of the growth of pathogenic and spoilage microorganisms. A slow rate of cooling of foods has been implicated as a major cause of foodborne diseases (Chapter 23). This can occur by trying to cool a large volume of hot or warm food in a big (deep) container in a refrigerator or overstuffing refrigerators with hot or warm foods. During thawing of a frozen food (such as an uncooked chicken), rapid thawing is desirable in order to control microbial growth, especially growth of pathogens. If the food is thawed slowly, the temperature on the food surface will soon increase, thereby allowing microbial growth, even when the inside is still frozen.

Refrigerated foods have limited shelf life, and, with time, microorganisms grow and spoil the products. In frozen foods, microorganisms (only cells, not spores) slowly die. However, even after long storage, some survive in frozen foods.

B. Nature of Food

Composition, pH, A_w, and presence of microbial inhibitors (preservatives) in a food can greatly influence growth, sublethal injury, and viability of microorganisms during storage at low temperature. A food with higher solid content (especially high proteins, carbohydrates, and lipids, but low ions), pH closer to 7.0, higher A_w, and the absence of microbial inhibitors facilitate growth and survival of microorganisms at refrigeration temperature and inflict less injury and cause less death at frozen temperature. Thus, the shelf life of refrigerated foods can be increased by using one or more of these factors, such as low pH, low A_w, incorporation of suitable microbial inhibitors, and, when possible, vacuum or modified air packaging (see Chapter 40).

In packaged frozen foods, ice may form in the packages (package ice), especially if the storage temperature fluctuates. During thawing, the ice melts and is absorbed by the food, resulting in an increase in the A_w in a localized area (e.g., in a bread) and making it susceptible to microbial growth after thawing.

C. Nature of Microorganisms

Although some microorganisms can grow at as low as –10°C, many mesophilic and thermophilic bacterial cells can be sublethally injured and may die with time at low temperatures above freezing. At temperatures below –10°C, vegetative cells of microorganisms can sustain sublethal injury and die. In general, Gram-negative or rod-shaped bacteria are more susceptible to the damaging effect of freezing than Gram-positive or spherical-shaped bacteria. Also, cells from the early exponential

phase of growth are more susceptible to freezing than those from the early stationary phase. Species and strains of microorganisms also differ greatly in sensitivity and resistance to freezing damage. Germination and outgrowth of spores of some *Clostridium* spp. can occur at as low as 2°C and maybe at a slightly higher temperature for some *Bacillus* spp. spores. Spores do not lose their viability in frozen foods. Some microbial enzymes, either heat stable or released by the dead and lysed cells, can catalyze reactions at temperatures above –20°C, but at a slow rate, and can reduce the acceptance quality of a food.[1–7]

V. METHODS

Foods are stored at low temperature in different ways to extend their shelf life. Many fresh fruits and vegetables are kept at temperatures between 10 and 20°C or lower, mainly to reduce their metabolic rate. Microorganisms to which these products are susceptible, namely yeasts and molds (and some bacteria), can grow at this temperature. Maintaining a low relative humidity to prevent moisture build-up on the food surface is very important to reduce their growth. Highly perishable products are generally stored at a low temperature, below 7ºC, often in combination with other preservation methods. The importance of rapid cooling of a food for microbiological safety has been mentioned before. The methods used for low-temperature preservation of foods and in food safety are briefly discussed.

A. Ice Chilling

This is used in retail stores where the foods are kept over ice; the surface in contact with the ice can reach between 0 and 1°C. Fresh fish, seafood, meats, cut fruits, vegetable salads (in bags), different types of ready-to-eat salads (prepared at the retail store), salad dressing (high pH, low caloric), sous vide, and some ethnic foods (e.g., tofu) are stored by this method. The trend is increasing.

Temperature fluctuation (due to the size of the container or melting of ice), duration of storage (fresh or several days), and cross-contamination (raw fish, shrimp, oysters, and ready-to-eat fish salads in an open container in the same display case at the retail store) can cause microbiological problems, especially from foodborne pathogens.

B. Refrigeration

The temperature specification for refrigeration of foods has changed over time (Chapter 20). Previously, 7°C (≅45°F) was considered a desirable temperature. However, technological improvements have made it economical to have domestic refrigeration units at 4 to 5°C (40 to 41°F). For perishable products, ≤4.4°C (40°F) is considered a desirable refrigeration temperature. Commercial food processors may use as low as ≅1°C for refrigeration of perishable foods (such as fresh meat and fish). For optimum refrigeration in commercial facilities along with low temperature, the relative humidity and proper spacing of the products are also controlled.

Raw and processed foods of plant and animal origin, as well as many prepared and ready-to-eat foods, are now preserved by refrigeration. Their volume is increasing rapidly because consumers prefer such foods. Some of these foods are expected to have a storage life of 60 d or more.

For refrigerated products expected to have a long shelf life, additional preservation methods are combined with the lowest possible temperature that can be used (close to –1°C). However, as the products are nonsterile, even a very low initial microbial population (e.g., ≤10 cells or spores per 10 g), capable of growing (or germinating) under the storage condition, can multiply to reach hazard (for pathogen) or spoilage levels, thereby reducing the safety and stability of the product. Any fluctuation in temperature or other abuse (e.g., a leak in a vacuum or modified atmosphere package, or oxygen permeation through the packaging materials) can greatly accelerate their growth. The processing and storage conditions may provide environments in which different types of spoilage and pathogenic microorganisms grow advantageously. This may increase spoilage and wastage of foods unless appropriate control measures are installed quickly.

C. Freezing

The minimum temperature used in home freezers (in the refrigerator) is –20°C, a temperature at which most of the free water in a food remains in a frozen state. Dry ice (–78°C) and liquid nitrogen (–196°C) can also be used for freezing; they are used for rapid freezing (instant freezing) and not for only freezing a food to that low temperature. Following freezing, the temperature of the foods is maintained ca. –20 to –30°C. Depending on the type, foods can be stored at refrigerated temperature for months or even more than a year. Raw produce (vegetables, fruits), meat, fish, processed products, and cooked products (ready-to-eat after thawing and warming) are preserved by freezing.

Microorganisms do not grow at –20°C in frozen foods. Instead, microbial cells die during frozen storage. However, the survivors can multiply in the unfrozen foods. Accidental thawing or slow thawing can facilitate growth of survivors (spoilage and pathogenic microorganisms). Spores can also germinate and outgrow, depending on the temperature and time following thawing. Enzymes, released by the dead microbial cells can reduce the acceptance quality of the food.[1–5]

VI. CONCLUSION

Microbial growth in a food depends on the storage temperature and the microbial types. Based on these two factors and the specific need, foods are stored below room temperature, refrigerated or frozen. Although microorganisms cannot grow in frozen foods, some can grow in refrigerated foods. In addition to preventing and retarding growth, microbial cells are also injured and killed at frozen and refrigerated temperatures. Because refrigeration maintains the acceptance quality of foods, lower temperature ($\simeq$ –1° to 5°C) is used in combination with other factors (low A_w, pH, etc.) to extend the shelf life of foods for 60 d or more. Like low temperature, reduced A_w is also used to control microbial growth in food and is discussed in Chapter 34.

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QUESTIONS

1. List the microbiological objectives of low-temperature preservation of food. How do these objectives differ from those for food preserved by heat?
2. Briefly discuss the mechanisms of microbial control by reducing the temperature of a food to 10ºC, to –1ºC, to –10ºC, and to –20ºC.
3. List the major factors that need to be considered for effective control of microorganisms in a food at low temperature, and briefly discuss the importance of each.
4. Discuss the microbial implications of the following in low-temperature preservation of foods: (a) fluctuation of storage (refrigerated and frozen) temperature, (b) slow cooling of a warm food, and (c) slow thawing of a frozen food.
5. Briefly discuss the microbiological problems of foods stored by chilling, refrigeration, and freezing.
6. List the psychrotrophic pathogens that can cause food hazard in refrigerated ready-to-eat foods and suggest a method that can be used to overcome the problem (consult Chapter 40).

CHAPTER 34

Control by Reduced A_w

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I. INTRODUCTION

The ability of dried seeds, grains, tubers, and fruits to resist spoilage was probably recognized by humans even before their discovery of agriculture. Subsequently, this simple method (drying) was practiced to preserve the large volume of foods produced during growing seasons to make them available during nongrowing seasons. Later, reduced A_w was also extended to preserve other foods (e.g., meat, fish, and milk) not only by removing water by drying but also by adding solutes (e.g., salt, honey, and starch) to bind water.

Since the beginning, reduced A_w has been used throughout human civilization in many ways not only to preserve foods and stabilize the food supply but also to develop different types of shelf-stable foods. Some of these include salted fish and meats; semidry and dry fermented sausages; dried fish, meat, vegetables, and fruits; evaporated and sweetened condensed milk; and dry milk, cheeses, bread and bakery products, flour, cereals, molasses, jams and jellies, chocolate, noodles, crackers, dried potatoes, dried eggs, and confectioneries. In more recent years, new technologies have helped produce foods with low A_w by freeze-drying, puffed-drying, freeze-concentration, and osmotic-concentration methods. In addition, a better understanding of the relationship and influence of moisture and A_w on microbial growth has been instrumental in producing many types of intermediate-moisture ready-to-eat foods. Efforts are also in progress to use low A_w along with other microbial control parameters, such as low pH, vacuum packaging, and low heat, to produce ready-to-eat meat, fish, and dairy products that can be stored at ambient temperature for relatively long periods of time. Because of their convenience, these foods are popular; the trend shows that the popularity will continue.

II. OBJECTIVES

The main objectives of reducing A_w in food are to prevent or reduce the growth of vegetative cells and germination and outgrowth of spores of microorganisms.[1–3] Prevention of toxin production by toxigenic molds and bacteria is also an important consideration. Microbial cells (not spores) also suffer reversible injury and death in foods with low A_w, although not in a predictable manner as in heat treatment. Finally, reduced A_w is also used to retain viability of starter-culture bacteria for use in food bioprocessing, which is discussed in Chapter 13. In this chapter, preservation of food by controlling microbial growth at low A_w is described.

III. MECHANISM OF ANTIMICROBIAL ACTION

Microorganisms need water for transport of nutrients, nutrient metabolism, and removal of cellular wastes. In a food, the total water (moisture) is present as free water and bound water; the latter remains bound to hydrophilic colloids and solutes (it can also remain as capillary water or in a frozen state as ice crystals) and is not available for biological functions. Thus, only the free water (which is related to A_w) is important for microbial growth. Microorganisms also retain a slightly lower A_w inside the cells than the external environment to maintain turgor pressure, and this is important for cell growth. If the free water in the environment is reduced either by removing water or by adding solutes and hydrophilic colloids, which cannot readily enter the cells, the free water from the cells flows outside in an effort to establish equilibrium. The loss of water causes an osmotic shock and plasmolysis, during which the cells do not grow. The water loss can be quite considerable even with a slight reduction in A_w. A 0.005 reduction in A_w from 0.955 to 0.950 in the environment reduces the intracellular water content by 50% in *Staphylococcus*

aureus and reduces the cell volume by 44% in *Salmonella* Typhimurium. Hence even a slight reduction in A_w, necessary for minimal growth of a microbial species or strain, prevents its growth. Unless a microbial cell regains its intracellular turgor by reducing internal A_w, it will either remain dormant or die. This is often the case with microorganisms sensitive to slight A_w reduction. However, some other microorganisms have developed very effective mechanisms such as transporting solutes inside or metabolizing solutes to overcome plasmolysis and regain turgor. Microorganisms that are relatively resistant to a great reduction in A_w and grow at relatively lower A_w have this capability.[1–3]

IV. INFLUENCING FACTORS

A. Nature of Process

Water activity and the total amount of water (% moisture) a food contains are different. The A_w of a food indicates the amount or fraction of the total amount of water available for some chemical or biochemical reactions. In pure water, both values are the same, but in food, A_w is always less than the total amount of water. Under a set of conditions, the relationship between the moisture content and the A_w of a food can be determined from the sorption isotherm. However, instead of a single line, the sorption isotherm forms a loop (hysteresis loop), depending on whether it is determined during removal of water from (desorption) or during addition of water to (adsorption) a food (see Figure 6.1). At the same moisture level, the A_w value obtained by desorption is lower than that obtained by adsorption. In controlling microbial growth by reducing A_w, this is quite important.

Solutes differ in their ability to reduce A_w. The amounts (% w/w) of NaCl, sucrose, glucose, and inverted sugar required to reduce A_w at 25ºC of pure water to 0.99 are 1.74, 15.45, 8.9, and 4.11 g; and to 0.92, they are 11.9, 54.34, 43.72, and 32.87 g, respectively. These solutes do not freely enter the microbial cells and thus have a greater inhibitory effect on microbial cells as compared with solutes that enter freely in cells (e.g., glycerol), which are required in higher amounts for similar inhibition.

Although microorganisms can be injured and die at reduced A_w, a lower A_w value is less detrimental. For a 90% reduction of salmonellae population in a product at 15ºC, it took 27 d at an A_w of 0.71, but 67 d at an A_w of 0.34.

Studies on minimal A_w values to support the growth of specific microorganisms have generated conflicting data. This could be due to the inherent problems with different techniques used to measure A_w. However, with the modern electronic hygrometers, this problem is expected to be minimized.[1–3]

B. Nature of Foods

Minimal A_w values for growth of microorganisms, as well as influence of A_w on viability loss, vary with the food characteristics and the food environment. In a homogeneous food, A_w will remain unchanged provided other factors do not change. However, a heterogenous food with ingredients or items of different A_w (e.g., a

sandwich or a meal with different items in the same package) will generate a gradient. This can lead to microbial growth in an item preserved by reduced A_w alone and stored with an item of high A_w containing a preservative. Also, condensation of water during storage with temperature fluctuation, followed by dripping of moisture in some areas of food, can alter a safe A_w level in these areas to an unsafe state.

The minimum A_w for growth of microorganisms in a food can be higher than that in a broth. Thus, *Sta. aureus* has a minimal A_w of growth of 0.86 in a broth, but it does not grow in shrimp at an A_w of 0.89. As the A_w is reduced, anaerobic bacteria will require more of an anaerobic environment for growth. *Clostridium perfringens* grew in a broth with an A_w of 0.995 at an O–R potential of +194 mV; when the A_w was reduced to 0.975, an O–R potential of +66 mV was required for growth. As the incubation temperature is moved in either direction from optimum without changing the A_w, the microorganisms require a longer time to grow. In a broth of A_w of 0.975, a *Clo. botulinum* E strain grew in 6 d at 30ºC, in 19 d at 15ºC, and in 42 d at 7.2ºC. The minimum A_w for growth of *Sta. aureus* was 0.865 at 30ºC, but changed to 0.878 at 25ºC. Reduced A_w and low pH interact favorably in inhibiting microbial growth. A *Clo. botulinum* B strain grew at an A_w of 0.99 up to pH 5.3 and at an A_w of 0.97 up to pH 6.0, but at an A_w of 0.95, it failed to grow even at pH 7.0. Similarly, a spoilage strain of *Clo. butyricum* grew at an A_w of 0.98 up to pH 3.8, but at an A_w of 0.97 failed to grow at pH 4.5 even after 30 d at 30ºC. Many chemical preservatives enhance the inhibitory effect of lower A_w on microbial growth. In the presence of low concentrations of sorbate, citrate, and phosphate, different microorganisms were found not to grow at the lowest A_w in which they grew in the absence of these chemicals.

Food composition can influence microbial death rate even at the same A_w. At an A_w of 0.33, *Escherichia coli* counts reduced by $\log_{10}2.8$ in ice cream powder, $\log_{10}4.8$ in dried potatoes, but by more than $\log_{10}6$ in coffee. Under the same conditions, the death rate of *Enterococcus faecalis* was much less. Although the survivors remain dormant in a low-A_w food, as soon as it is rehydrated, the microorganisms regain the ability to metabolize and multiply. Thus, a rehydrated food should be treated as a perishable food that, unless effective control methods are used, can be unsafe and spoiled.[1–3]

C. Nature of Microorganisms

Microorganisms differ greatly in their minimal A_w requirement for growth, sporulation, and germination (Table 34.1). In general, molds and yeasts can grow at lower A_w values than bacteria; among pathogenic and spoilage bacteria, Gram-negatives require a slightly higher A_w than Gram-positives for growth. *Sta. aureus*, however, can grow at an A_w of 0.86. Sporulation by sporeforming bacteria occurs at A_w values in which the species and strains grow, whereas germination may occur at slightly lower A_w values. Toxin production may occur at the A_w of growth (by *Clo. botulinum*) or at a slightly higher than minimum A_w of growth (by *Sta. aureus* at 0.867 at 30ºC and 0.887 at 25ºC). The minimum A_w for microbial growth can vary with the type of solutes in a food. *Clo. botulinum* Type E failed to grow below an A_w of 0.97 when NaCl was used as a solute, but grew up to an A_w of 0.94 when glycerol was the

Table 34.1 Minimum A_w for Microbial Growth at Optimum Growth Temperature

Microorganism	A_w
Bacteria	
Bacillus cereus	0.95
Bacillus stearothermophilus	0.93
Clostridium botulinum Type A	0.95
Clostridium botulinum Type B	0.94
Clostridium botulinum Type E	0.97
Clostridium perfringens	0.95
Escherichia coli	0.95
Salmonella spp.	0.95
Vibrio parahaemolyticus	0.94
Staphylococcus aureus	0.86
Pseudomonas fluorescens	0.97
Lactobacillus viridescens	0.94
Yeast	
Saccharomyces cerevisiae	9.90
Saccharomyces rouxii	0.62
Debaryomyces hansenii	0.83
Molds	
Rhizopus nigricans	0.93
Penicillium chrysogenum	0.79
Penicillium patulum	0.81
Aspergillus flavus	0.78
Aspergillus niger	0.77
Alternaria citri	0.84

solute. *Pseudomonas fluorescens* similarly showed a minimum A_w of 0.957 for growth with NaCl but 0.94 with glycerol. This is because glycerol enters freely inside the cell and thus does not cause osmotic stress as nonpermeable NaCl, sucrose, and similar solutes. Germination of spores in glycerol occurs at lower A_w because *Clo. botulinum* E spores, with glycerol, germinate at an A_w of 0.89, but do not grow below an A_w of 0.94. The growth rate of microorganisms also decreases as the A_w value is lowered. The growth rate of *Sta. aureus* reduces to about the 10% level at an A_w of 0.90 of its optimum growth rate at an A_w of 0.99.

Among microbial groups, halophiles, osmophiles, and xerophiles grow better or grow preferentially at lower A_w values. Halophiles, such as some vibrios, need NaCl in varying amounts for growth. Osmophiles and xerophiles, yeasts, and molds grow at an A_w less than 0.85, as they do not have competition from bacteria.

Optimum growth of most microorganisms in foods occurs at an $A_w \geq 0.98$. At $A_w \geq 0.98$, Gram-negatives, having a faster growth rate, predominate if other needs for optimum growth are fulfilled. As the A_w drops to 0.97, Gram-positive bacteria, such as bacilli, lactobacilli, micrococci, and clostridia, predominate. Below an A_w of 0.93, Gram-positive bacteria, such as micrococci, staphylococci, enterococci, and pediococci, as well as yeasts and molds grow preferentially. As the A_w drops below 0.86, osmophilic yeasts and xerophilic molds predominate. They can grow in foods with an A_w up to 0.6; as A_w drops below 0.6, microbial growth stops.[1–3]

V. METHODS

The water activity of foods can be reduced by using one or more of these three basic principles: (a) removing water by dehydration, (b) removing water by crystallization, or (c) adding solutes to bind water. Some of these methods and their effect on microorganisms are briefly described.[1–5]

A. Natural Dehydration

Natural dehydration is a low-cost method in which water is removed by the heat of the sun. It is used to dry grains as well as to dry some fruits (raisins), vegetables, fish, meat, milk, and curd (from milk), especially in warmer countries. The process is slow; depending on the conditions used, spoilage and pathogenic bacteria as well as yeasts and molds (including toxigenic types) can grow during drying.

B. Mechanical Drying

Mechanical drying is a controlled process, and drying is achieved in a few seconds to a few hours. Some of the methods used are tunnel drying (in which a food travels through a tunnel against flow of hot air that removes the water), roller drying (in which a liquid is dried by applying a thin layer on the surface of a roller drum heated from inside), and spray drying (in which a liquid is sprayed in small droplets, which then come in contact with hot air that dries the droplets instantly). Vegetables, fruits, fruit juices, milk, coffee, tea, and meat jerky are some foods that are dried by mechanical means. Liquids may be partially concentrated before drying by evaporation, reverse osmosis, freeze-concentration, and addition of solutes.

Depending on the temperature and time of exposure, some microbial cells can die during drying, whereas other cells can be sublethally injured. Also, during storage, depending on the storage conditions, microbial cells can die rapidly at the initial stage and then at a slow rate. Spores generally survive and remain viable during storage in a dried food.

C. Freeze-Drying

The acceptance quality of food is least affected by freeze-drying, as compared with both natural and mechanical drying. However, freeze-drying is a relatively costly process. It can be used for both solid and liquid foods. The process initially involves freezing the food, preferably rapidly at a low temperature, and then exposing the frozen food to a relatively high vacuum environment. The water molecules are removed from the food by sublimation (from solid state to vapor state) without affecting its shape or size. The method has been used to produce freeze-dried vegetables, fruits, fruit juices, coffee, tea, and meat and fish products, some as specialty products. Microbial cells are exposed to two stresses — freezing and drying — that reduce some viability as well as induce sublethal injury. During storage, especially at a high storage temperature and in the presence of oxygen, cells die rapidly initially and then more slowly. Spores are not affected by the process.

D. Foam-Drying

The foam-drying method consists of whipping a product to produce a stable foam to increase the surface area. The foam is then dried by warm air. Liquid products, such as egg white, fruit purees, and tomato paste, are dried in this manner. The method itself has very little lethal effect on microbial cells and spores. However, a concentration method before foaming, the pH of the products, and low A_w cause both lethal and reversible damages to microbial cells.

E. Smoking

Many meat and fish products are exposed to low heat and smoke for cooking and depositing smoke on the surface at the same time. The heating process removes water from the products, thereby lowering their A_w. Many low-heat-processed meat products (dry and semidry sausages) and smoked fishes are produced this way. Heat kills many microorganisms. The growth of the survivors is controlled by low A_w as well as the many types of antimicrobial substances present in the smoke.

F. Intermediate Moisture Foods

Intermediate moisture foods (IMF) that have A_w values of ca. 0.70 to 0.90 (with moisture contents of ca. 10 to 40%). They can be eaten without rehydration, but are shelf-stable for a relatively long period of time without refrigeration and considered microbiologically safe. Some of the traditional IMFs includes salami, liverwurst, semidry and dry sausages, dried fruits, jams and jellies, and honey. However, in recent years, many other products have been developed, such as pop tarts, slim jims, ready-to-spread frosting, breakfast squares, soft candies, fruit rolls, food sticks, and soft granola bars. The low A_w value and relatively high moisture is obtained by adding water-binding solutes and hydrophilic colloids. Microorganisms can survive in the products, but because of low A_w, bacteria cannot grow. However, yeasts and molds can grow in some. To inhibit their growth, specific preservatives, such as sorbate and propionate, are added.[4,5]

VI. CONCLUSION

The A_w of a food is directly proportional to the level of free water it contains. As microbial growth is adversely affected even with a slight reduction in A_w, it has been used, where possible, to retard and prevent growth mainly of spoilage bacteria in food. It is achieved either by removing or binding the free water. At lower A_w, microbial cells can lose viability and become injured with time. However, this alone cannot be used to ensure safety of food from pathogens. Although as a single method it has limited application in food preservation, along with other methods, such as low temperature or low pH, it can be used to extend the shelf life of food very effectively. The effectiveness of low pH, either alone or with other methods, in controlling microorganisms in food is discussed in Chapter 35.

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QUESTIONS

1. List the microbiological objectives of reducing A_w in foods.
2. Discuss the mechanisms by which low A_w produces an antimicrobial effect.
3. Define sorption isotherm. Describe the hysteresis loop, and discuss how there can be problems in selecting the correct A_w in a food from the hysteresis loop.
4. Discuss the influences of different solutes in lowering the A_w value of a food and controlling microbial growth. (Use NaCl, sucrose, and glycerol as examples.)
5. Briefly describe the interaction of A_w with other intrinsic and extrinsic factors in controlling microorganisms in a food.
6. List how different microorganisms are controlled in a food as the A_w value drops from 0.99 to 0.60. Discuss the minimal A_w for growth, toxin production, sporulation, and germination by a bacterial species (*Clo. botulinum*).
7. Briefly explain the microbiological concerns of naturally dried foods, freeze-dried foods, smoked foods, rehydrated foods, and IMFs.
8. Briefly discuss how in the rural areas in many developing countries lower A_w along with lower pH and a suitable preservative can be used to extend shelf life of some perishable foods (use one example) at room temperature (see Chapter 40).

CHAPTER 35

Control by Low pH and Organic Acids

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I. INTRODUCTION

During the early stages of human history when food was scarce, our ancestors probably recognized that some foods from plant sources, especially fruits, resisted spoilage. Later, they observed that the fermented foods and beverages prepared from fruits, vegetables, milk, fish, and meat were much more shelf stable than the raw materials from which they were produced. That probably provided an incentive to

develop large varieties of fermented foods, especially in tropical areas where, unless preserved, many foods spoil rapidly.[1]

When the microbial involvement in food spoilage and foodborne diseases was recognized, methods to control their growth as well as to kill them in food were studied. It was observed that over a restricted pH range, many microorganisms present in food can grow, but at lower pH ranges many of them die. Once this was recognized, many organic acids were used as food additives. In addition to their effectiveness as food preservatives, they are also used to improve acceptance qualities of foods. The amounts and types of organic acids that can be added to foods are governed by regulatory agencies.

Organic acids can be present in foods in three ways. They can be present naturally, such as citric acid in citrus fruits, benzoic acid in cranberries, and sorbic acid in rowan berries. Some, such as acetic, lactic, and propionic acids, are produced in different fermented foods by desirable food-grade starter-culture bacteria. Many acids are also added to foods and beverages to reduce the pH. Among the organic acids used in food as preservatives are acetic, propionic, lactic, citric, sorbic, and benzoic, their salts, and some derivatives of benzoic acid (e.g., paraben). The influence of these acids in reducing food pH and producing antimicrobial effects are briefly discussed in this chapter.

II. OBJECTIVES

The major antimicrobial objective of using weak organic acids is to reduce the pH of food to control microbial growth.[1] As the pH drops below 5.0, some bacteria become injured and die. However, the death rate in low pH is not predictable as in the case of heat. Thus, it could not be used with the objective of destroying a predictable percentage of a microbial population in the normal pH range of foods.[1–6]

III. MECHANISMS OF ANTIMICROBIAL ACTION

The antimicrobial action of a weak organic acid is produced by the combined actions of the undissociated molecules and the dissociated ions.[1–6] Microorganisms that are important in food tend to maintain an internal cytoplasmic pH ca. 6.5 to 7.0 in acidophiles and 7.5 to 8.0 in neutrophiles. The internal pH (pHi) is tightly regulated and drops by ca. 0.1 unit for each 1.0 unit change in the environmental pH (pHo). For nutrient transport and energy synthesis, the microorganisms also maintain a transmembrane pH gradient (ca. 0.5 to 1.0 unit with alkaline pHi) and a proton gradient (ca. 200 mV); together, they form the proton motive force (PMF).

When a weak organic acid is added to the environment (in a food), depending on the pH of the food, the pK of the acid, and the temperature, some of the molecules dissociate whereas others remain undissociated (Table 35.1). At the pH of most foods (pH 5 to 8), except for paraben, the organic acid molecules remain generally dissociated; as a result, $[H^+]$ in the environment increases, which interferes with the transmembrane proton gradient of microbial cells. To overcome this, the cells trans-

Table 35.1 Influence of pH on the Amount (%) of Dissociated Ions of Weak Organic Acids

Acid	pK	% Dissociated at pH		
		4	5	6
Acetic	4.8	15.5	65.1	94.9
Propionic	4.9	12.4	58.3	93.3
Lactic	3.8	60.8	93.9	99.3
Citric	3.1	81.1	99.6	>99.1
Sorbic	4.8	18.0	70.0	95.9
Benzoic	4.2	40.7	87.2	98.6
Paraben[a]	8.5	<0.1	0.1	0.3

[a] Paraben: esters of benzoic acids.

port protons through the proton pump, which causes depletion in energy and a decrease in pHi. The structures on the cell surface, outer membrane or cell wall, inner membrane or cytoplasmic membrane, and periplasmic space are also exposed to $[H^+]$. This can adversely affect the ionic bonds of the macromolecules and thus interfere with their three-dimensional structures and some related functions. At pH < 5.0, the undissociated molecules of some acid can be considerably high. Being lipophilic (except citric), they enter freely through the membrane as a function of the concentration gradient. Because the pHi is much higher than the pK of the acid, the molecules dissociate, releasing protons and anions. Some anions (e.g., acetate and lactate) are metabolized by several microorganisms as a C source. If they are not metabolized, the anions are removed from the cell interior. However, the $[H^+]$ will reduce the internal pH and adversely affect the proton gradient. To overcome this problem, the cells pump out the excess protons by expending energy. At lower pHo (pH 4.5 or below), this represents an expense of a large amount of energy that cells may not be able to generate. As a result, the internal pH drops, adversely affecting the pH gradient. The low pH can also act on the cellular components (such as proteins) and adversely affect their structural (by interfering with the ionic bonds) and functional integrity.[1,3,4,7]

These changes can interfere with the nutrient transport and energy generation, and in turn interfere with microbial growth. In addition, low pH can reversibly and irreversibly damage cellular macromolecules, which can subsequently inflict sublethal as well as lethal injury to cells.

Low pH can alter the ionic environment of the spore coat by replacing its ions (e.g., Ca^{2+}) with H^+ and make the spores unstable toward other environmental stresses, such as heat and low A_w.[1–6]

IV. INFLUENCING FACTORS

A. Nature of Acids

The weak organic acids used in food vary in antimicrobial effectiveness because of their differences in pK. An acid with higher pK has proportionately higher amounts of undissociated molecules at a food pH and is more antimicrobial. Limited studies

have revealed that, in general, under similar conditions, the antimicrobial effectiveness of four acids follows the order acetic > propionic > lactic > citric. Similarly, at lower pH and higher concentrations, an acid is more antimicrobial. Solubility of the acids in water is also important for the desirable effect. Acetate, propionate, lactate, and citrate are very soluble in water, whereas benzoate (50 g%), sorbate (0.16 g%), and paraben (0.02 to 0.16 g%) are poorly soluble in water, and thus, at the same concentration, have different effectiveness. In many studies, the antimicrobial effectiveness of these acids against microorganisms is studied on a percent basis (g in 100 ml). However, they vary in molecular weight; thus, at the same concentration, they have different numbers of molecules and produce different concentrations of undissociated molecules as well as dissociated ions. For a comparison, it is better to use the acids on a molar concentration basis (see Chapter 16).

Organic acids also differ in their lipophilic properties, which in turn regulate their ease in entering the cells. Acetic and propionic acids are more lipophilic than lactic acid and have more antimicrobial effectiveness than lactic acid. Citrate is transported through the membrane by a specific transport mechanism (citrate permease) and is less effective than lipophilic acids. Many microorganisms can metabolize the anions of weak acids, such as acetate, lactate, and citrate. Use of salts of these acids may decrease the antimicrobial effect at higher pH. Some acids show synergistic effects when used in suitable combinations (e.g., acetic and lactic acids; propionic and sorbic acids) or with another preservative (e.g., benzoic acid with nisin; propionic, acetic, or lactic acid with nisin or pediocin AcH; propionate or benzoate with CO_2).[1,4,5,7]

B. Nature of Foods

The normal pH of foods varies greatly from a very acid range (3.0; citrus juice) to an alkaline range (pH 9.0; egg albumen). The initial pH can strongly influence the antimicrobial effect of an acid. An acid is more inhibitory in a food at a lower pH than in one at a higher pH. The buffering action of the food components also reduces the effectiveness of low pH. Nutrients can also facilitate repair of sublethal acid injury of microorganisms.

C. Nature of Microorganisms

Microorganisms important in food vary greatly in the lower limit of pH that allows growth (Table 35.2). In general, Gram-negative bacteria are more sensitive to low pH than are Gram-positive bacteria, and yeasts and molds are the least sensitive. Fermentative bacteria are more resistant to lower pH than are respiring bacteria, probably because they are able to resist changes in external pH as well as withstand slightly lower internal pH. The ability of yeasts and molds to withstand low pH is also due to these factors.[1,3,4,6] The antimicrobial property of an organic acid is enhanced by heat, low A_w, presence of some other preservatives, and low storage temperatures.

The inhibitory effect of weak acids is reduced at higher microbial load. Also, in a mixed microbial population, the metabolism of an acid (such as lactate) by one

Table 35.2 Minimum pH at which Growth Occurs

Microorganism	Minimum Growth pH
Gram-Negative Bacteria	
Escherichia coli	4.4
Pseudomonas spp.	5.6
Salmonella	4.5
Vibrio spp.	4.8
Serratia spp.	4.4
Gram-Positive Bacteria	
Bacillus cereus	4.9
Bacillus stearothermophilus	5.2
Clostridium botulinum	4.6
Clostridium perfringens	5.0
Enterococcus faecalis	4.4
Lactobacillus spp.	3.8
Staphylococcus aureus	4.0
Listeria monocytogenes	4.6
Yeasts	
Candida spp.	1.5 to 2.3
Saccharomyces spp.	2.1 to 2.4
Hansenula spp.	2.1
Rhodotorula spp.	1.5
Molds	
Aspergillus spp.	1.6
Penicillium spp.	1.6 to 1.9
Fusarium spp.	1.8

resistant species can reduce its effective concentration against another sensitive species, allowing the latter to grow. Some microorganisms important in food, such as some *Salmonella* strains, seem to have genetic determinants that enable them to grow at higher acid concentrations (or lower pH) than other strains of the same species. The acid tolerance seems to be related to overproduction of a group of proteins (stress proteins) by these strains (see Chapter 9).

Finally, microorganisms differ in their sensitivity to different organic acids. Yeasts and molds are particularly sensitive to propionic and sorbic acids, and bacteria are more sensitive to acetic acid. Bacterial spores at lower pH become susceptible to heat treatment and do not germinate and outgrow at minimum A_w of growth. Also, the inhibitory effect of NO_2 against spores is more pronounced at the lower pH range of growth.

V. ACIDS USED

A. Acetic Acid

Acetic acid is used usually as vinegar (5 to 10% acetic acid) or as salts of sodium and calcium at 25% or higher levels in pickles, salad dressings, and sauces. It is more effective against bacteria than yeasts and molds. Bacteria that grow better

above pH 6.0 are more inhibited. The inhibitory concentrations of undissociated acid are 0.02% against *Salmonella* spp., 0.01% against *Staphlococcus aureus*, 0.02% against *Bacillus cereus*, 0.1% against *Aspergillus* spp., and 0.5% against *Saccharomyces* spp. The inhibitory action of acetic acid is produced through neutralizing the electrochemical gradient of the cell membrane as well as denaturing proteins inside the cells. Besides its use in food, acetic acid has been recommended for use (1 to 2%) in carcass wash to reduce bacterial levels.[1,3,7]

B. Propionic Acid

Propionic acid is used as salts of calcium and sodium at 1000 to 2000 ppm (0.1 to 0.2%) in bread, bakery products, cheeses, jam and jellies, and tomato puree. It is effective against molds and bacteria but almost ineffective against yeasts at concentrations used in foods. The inhibitory concentration of undissociated acid is 0.05% against molds and bacteria. The antimicrobial action is produced through the acidification of cytoplasm as well as destabilization of membrane proton gradients.[1,3,7]

C. Lactic Acid

Lactic acid is used as acid or sodium and potassium salts up to 2% in carbonated drinks, salad dressings, pickled vegetables, low-heat-processed meat products, and sauces. It is less effective than acetic, propionic, benzoic, or sorbic acids, but more effective than citric acid. It is more effective against bacteria but quite ineffective against yeasts and molds. It produces an inhibitory effect mainly by neutralizing the membrane proton gradient. The sodium salt of lactic acid may also reduce A_w. L(+)-lactic acid is preferred over D(–)-lactic acid as a food preservative. It has also been recommended at a 1 to 2% level to wash carcasses of food animals to reduce microbial load.[1,3,7]

D. Citric Acid

Citric acid is used at 1% (or more) in nonalcoholic drinks, jams and jellies, baking products, cheeses, canned vegetables, and sauces. It is less effective than lactic acid against bacteria as well as yeasts and molds. Its antibacterial effect is probably by a mechanism different than that for lipophilic acids. The antibacterial effect is partially due to its ability to chelate divalent cations. However, many foods can have sufficient divalent cations to neutralize this effect.[3,7]

E. Sorbic Acid

It is an unsaturated acid and used either as acid or as salts of sodium, potassium, or calcium. It is used in nonalcoholic drinks, some alcoholic drinks, processed fruits and vegetables, dairy desserts, confectioneries, mayonnaise, salad dressings, spreads, and mustards. The concentrations used vary from 500 to 2000 ppm (0.05 to 0.2%). It is more effective against molds and yeasts than against bacteria. Among bacteria, catalase-negative (e.g., lactic acid bacteria) are more resistant than catalase-positive

species (e.g., aerobes, *Sta. aureus,* and *Bacillus* spp.). Also, aerobic bacteria are more sensitive to it than anaerobic bacteria. The inhibitory concentrations of undissociated acid are as follows: ≤0.01% (100 ppm) for *Pseudomonas* spp., *Sta. aureus, Esc. coli,* and *Serratia* spp.; ≅0.1% for *Lactobacillus* spp. and *Salmonella* spp.; and ≤0.02% for most yeasts and molds, but ≅1.0% for *Clostridium* spp.

The antimicrobial effect of sorbate is produced through its inhibitory action on the functions of some enzyme, some from the citric acid cycle. It also interferes with synthesis of cell wall, protein, RNA, and DNA. Also, like other organic acids, it interferes with the membrane potential and inhibits spore germination.[3,5,8]

F. Benzoic Acid

Benzoic acid is used as an acid or sodium salt at 500 to 2000 ppm (0.05 to 0.2%) in many low-pH products, such as nonalcoholic and alcoholic beverages, pickles, confectioneries, mayonnaise and salad dressings, mustards, and cottage cheese. It is more effective against yeasts and molds than bacteria. The inhibitory effect is produced by both the undissociated and dissociated acids. The inhibitory concentrations of undissociated acid are 0.01 to 0.02% against bacteria and 0.05 to 0.1% against yeasts and molds. The inhibitory action is produced in several ways. It inhibits the functions of many enzymes necessary for oxidative phosphorylation. Like other acids, it also destroys the membrane potential. In addition, it inhibits functions of membrane proteins.[3,5,7]

G. Parabens (Esters of *p*-Hydroxybenzoic Acid)

Parabens are used as methyl, ethyl, butyl, or propyl parabens. They are broad-spectrum antimicrobial agents. Because of high pK values, they are effective at high pH and against bacteria, yeasts, and molds. They are used at 100 to 1000 ppm (0.01 to 0.1%) in nonalcoholic and alcoholic beverages, fruit fillings, jams and jellies, pickles, confectioneries, salad dressings, spreads, and mustards. The undissociated inhibitory concentrations are 0.05 to 0.1% against yeasts, molds, and bacteria. Propyl- and butyl parabens are more inhibitory than the others.

Parabens produce antimicrobial action by acting on several targets of microbial cells. They may inhibit functions of several enzymes. They dissolve in membrane lipids and interfere with membrane functions, including transport of nutrients. They also interfere with the synthesis of proteins, RNA, and DNA. In addition, they destroy the membrane potential as other weak organic acids do.[3,5,7]

VI. CONCLUSION

A pH lower than the minimum growth pH prevents microbial growth by affecting energy production, enzymatic activity, transportation of nutrients, and others. In addition to controlling growth, microbial cells are sublethally injured or killed by low pH. Many food-grade organic acids along with other methods are used to control microbial growth in food. Low pH is also used to prevent germination of bacterial

spores in food. Although many Gram-negative pathogenic bacteria are very sensitive to low pH, it cannot be used to eliminate these pathogens during storage of food. The fact that some strains can become acid resistant has created concern on such a practice in the processing and preservation of some low-pH foods. In addition to low pH and the methods discussed before, low O–R potential is used to control microbial growth in food, and is discussed in Chapter 35.

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QUESTIONS

1. Discuss the mechanisms of antimicrobial action of weak organic acids and low pH. Explain the differences in sensitivity to low pH among Gram-negative and Gram-positive bacteria, bacterial spores, yeasts, and molds.
2. Define the pK of an organic acid and, using acetic and lactic acids as examples, describe their differences in antibacterial effect at pH 5 and 6.
3. Briefly discuss the influence of the following factors on antimicrobial effectiveness of low pH: acids, foods, and microorganisms.
4. List the specific acids to be used to inhibit growth of Gram-positive bacteria, Gram-negative bacteria, yeasts, and molds.
5. Discuss the antimicrobial properties of acetic acid, propionic acid, lactic acid, benzoic acid, and parabens.
6. Some low-pH foods, such as apple juice, orange juice, and fermented sausage, have recently been implicated in foodborne disease outbreaks by strains of *Esc. coli* O157:H7, *Salmonella,* and *Listeria monocytogenes*. Explain the possible reasons and suggest methods that could be used to reduce this problem.

CHAPTER 36

Control by Modified Atmosphere (or Reducing O–R Potential)

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I. INTRODUCTION

Humans probably recognized in the early ages of agriculture and animal husbandry that many foods were susceptible to quality loss when stored in air. This probably led to the preservation of foods ca. 6000 B.C. by initially excluding air in pits and, later, with the invention of pottery and baskets, in large vessels and containers. Storage of grains, semidry products, some fermented products, concentrated syrups and molasses, and similar products are still stored in large, closed, air-tight vessels in many parts of the world to prevent insect infestation and the growth of molds and yeasts. However, this method of changing the gaseous environment to preserve more perishable products, such as fresh meat, fish, fruits, and vegetables and other

processed products, originated in the early 20th century. During the 1920s and 1930s, studies revealed that by using CO_2 in higher concentrations (4 to 100%), the growth of molds on fresh meats could be greatly reduced and ripening of fruits and vegetables could be prolonged. In the 1960s, refrigerated beef carcasses were transported by ship from New Zealand and Australia to other countries in controlled CO_2-rich environments.

In the 1960s, the availability of suitable plastic materials for packaging and the necessary technology helped develop methods to preserve foods in different-sized packages in an altered atmosphere. This was achieved either by vacuum packaging or by gas flushing the package with one gas or a mixture of gases. During storage, the gaseous environment could change because of the metabolic activities of the food products and microorganisms and also to the permeability of gasses from the air through the packaging materials.[1,2]

In recent years, modification of the storage environment to preserve foods has become one of the methods used most often. The desire of consumers for fresh foods, foods that have been given less processing treatments and without undesirable preservatives, as well as the availability of the necessary technology, have helped in the economical production of many convenient and ready-to-eat foods that are preserved by altering the environment. As the demand for such food is expected to grow in the future, the use of an altered atmosphere to preserve these foods will increase.[1,2]

Three terminologies are used to alter the atmosphere in foods in order to preserve and increase their acceptance quality. To avoid confusion, these terms are defined here:

1. *Controlled Atmosphere Packaging (CAP).* In this method, the atmosphere in a storage facility is altered and the levels of the gases are continually monitored and adjusted as required. This is expensive to operate and used for long-term storage of fruits and vegetables to maintain their freshness.
2. *Modified Atmosphere Packaging (MAP).* This method, unlike CAP, does not require a high degree of control of the gaseous environment during the entire storage period. In this method, a food is enclosed in a high gas-barrier packaging material; the air is removed from the package, which is then flushed with a particular gas or combination of gases; and the package is hermetically sealed.
3. *Vacuum Packaging.* This method involves removal of air from the package and then sealing the package hermetically.

MAP and vacuum packaging have been described as two separate methods by some, but as one method (e.g., MAP) by others. In this chapter, MAP has been used both for vacuum packaging and gas-flush packaging.

II. OBJECTIVES

The objectives of MAP are to control or reduce the growth of undesirable microorganisms in food. The technique also helps retard enzymatic and respiratory activities of fresh foods. The growth of aerobes (molds, yeasts, and aerobic bacteria) is

prevented in products that are either vacuum packaged or flushed with 100% CO_2, 100% N_2, or a mixture of CO_2 and N_2. However, under these conditions, anaerobic and facultative anaerobic bacteria can grow unless other techniques are used to control their growth.

III. MECHANISM OF ANTIMICROBIAL ACTION

The antimicrobial action in MAP foods can be produced by changes in the redox potential (Eh) and CO_2 concentrations based on the methods used. Aerobes and anaerobes, depending on the microbial species and reducing or oxidizing state of food, have different Eh requirements for growth, whereas facultative anaerobes grow over a wide Eh range. Vacuum packaging and gas flushing, especially with CO_2 or N_2 or their mixture and no O_2, discourage growth of aerobes but encourage growth of facultative anaerobes and anaerobes. However, even under these conditions of packaging, tissue oxygen and dissolved and trapped oxygen can allow initial growth of aerobes to produce CO_2 even by the proteolytic microbes. In addition, natural reducing components in foods, such as the –SH group in protein-rich foods, and ascorbic acid and reducing sugars in fruits and vegetable products, can alter the Eh of a food and encourage growth of anaerobes and facultative anaerobes. Thus, it is not possible to control microbial growth just by changing the Eh. Other methods, in addition to modification of environment, are necessary for effective preservation of foods. However, by controlling the growth of aerobic bacteria, many of which have short generation times, the shelf life of a product is greatly extended.

When CO_2 is used in high concentrations (20 to 100%) alone or in combination with N_2 or O_2, or both, the shelf life of MAP foods is also extended. Several mechanisms by which CO_2 increases the lag and exponential phases of microorganisms have been proposed, such as rapid cellular penetration of CO_2 and alteration in cell permeability, solubilization of CO_2 to carbonic acid (H_2CO_3) in the cell with the reduction of the pH inside the cells, and interference of CO_2 with several enzymatic and biochemical pathways, which in turn slow the microbial growth rate. The inhibitory effect of CO_2 on microbial growth occurs at the 10% level and increase with the increase in concentration. Too high a concentration can inhibit growth of facultative spoilage bacteria and stimulate growth of *Clostridium botulinum*.[2–6]

IV. INFLUENCING FACTORS

A. Nature of Process

These factors include efficiency of vacuum, permeability of packaging materials to O_2, and the composition of gas (in the gas-flushing method) used.[2–6] High vacuum can effectively control the growth of aerobes by removing O_2 from the products, except probably trapped and dissolved O_2. Similarly, packaging films that prevent

or considerably reduce permeation of O_2 during storage effectively control the growth of aerobes. Minute leaks in the film can adversely affect the protective effect of vacuum packaging or gas-flush packaging. The development of polymeric film has helped greatly reduce the O_2 permeability. In gas flushing, CO_2 and N_2, and in the case of fresh meat along with some O_2, are used in a mixture. N_2 is used as an inert filler and O_2 is used to give the red oxymyoglobin color of meat. CO_2 is used for its antimicrobial effect. As low as 20% CO_2 has been found to control growth of aerobes, such as *Pseudomonas, Acinetobacter*, and *Morexella*; however, in general, CO_2 at 40 to 60% gives better results. In some cases, 100% CO_2 is used.

B. Nature of Foods

The amounts of oxygen (dissolved and trapped), metabolizable carbohydrates, and other nutrients and the reducing components present influence the growth of microbial types in a MAP food. In the presence of O_2, aerobes such as *Pseudomonas* utilize glucose and lactate. Growth of facultative anaerobes, however, discourages the growth of aerobes but can stimulate the growth of anaerobes. Reducing components also encourage the growth of anaerobes. The presence of specific inhibitors, either present in a food or produced by associated bacteria (such as bacteriocins by some lactic acid bacteria), low A_w, and low pH also influence the microbial ability to grow or not grow under specific packaging conditions.

C. Nature of Microorganisms

Aerobes can have limited initial growth, depending on the oxygen present in a vacuum-packaged or gas-flushed-packaged product.[2–6] However, their growth will stop as soon as the facultative anaerobes start growing. These include lactic acid bacteria, *Brochothrix thermosphacta*, some *Enterobacteriaceae,* and *Corynebacteriaceae*. In low-pH products, a favorable growth of lactic acid bacteria can reduce or prevent growth of other bacteria because the former can produce acids and other antibacterial substances (e.g., bacteriocins). Also, the composition of the gas mixture influences the predominant types; with 100% CO_2, lactic acid bacteria, especially *Leuconostoc* and *Lactobacillus* spp., predominate. CO_2 at ca. 20% concentration controls growth of *Pseudomonas* spp., but a CO_2 concentration of more than 60% is required to control growth of *Enterobacteriaceae*. As the Eh of a product starts reducing, the anaerobes, especially *Clostridium* spp., start growing. The presence of reducing agents in the food also favors growth of anaerobes. The possible growth of *Clo. botulinum* in MAP refrigerated foods, especially Type E and nonproteolytic Type B, is a major concern. Nonsporeforming psychrotrophic pathogens, especially the facultative anaerobes (*Listeria monocytogenes and Yersinia enterocolitica*) can also multiply in MAP foods. In addition, some mesophilic facultative anaerobic pathogens with growth capabilities at 10 to 12ºC (some *Salmonella* and *Escherichia coli* O157:H7 strains and *Staphylococcus aureus*) can also grow if the MAP foods are temperature abused during storage. Suitable preservatives, low pH, or low A_w, or their combination, should be used as additional hurdles for their control.[2–6]

V. METHODS

A. Vacuum Packaging

Vacuum packaging is predominantly used as retail packs in many fresh and ready-to-eat meat products, including beef, pork, lamb, chicken, and turkey. Red meat, due to change in color to purple (reduced myoglobin), is not very popular with consumers. The refrigerated storage life of these products varies greatly: ca. 3 to 4 weeks in fresh meat and as long as 8 weeks or more in processed meats. If the products have low A_w or low pH, or both, are produced under sanitary conditions, and kept at temperature ca.1.5ºC, the storage life can be much longer. In low-pH products, *Leuconostoc* spp. and *Lactobacillus* spp. usually predominate, whereas in high-pH products, *Bro. thermosphacta, Serratia liquifacience,* and *Hafnia* spp. are isolated, along with lactic acid bacteria. In fresh beef and pork and roasted beef, psychrotrophic *Clostridium* spp. have also been found to grow, even at temperatures below –1ºC. Vacuum packaging is also used in different types of cheeses, sausages, and low-pH condiments to control growth of yeasts and molds.[2–6]

B. Gas Flushing

The gas-flushing method is used in both bulk and retail packs to increase the shelf life of many refrigerated foods in European countries. In the U.S., it is used in products such as fresh pasta, bakery products, cooked poultry products, cooked egg products, fresh and cooked fish and seafood, sandwiches, raw meats, and some vegetables. The gases usually used are a mixture of CO_2 and N_2, with some O_2 for packaging red meats. Generally, the composition of gas mixtures must be tailored for each product. In raw meats, a composition of 75% CO_2, 15% N_2, and 10% O_2 was found to effectively prevent growth of *Pseudomonas fragi* for a limited period. Products flushed with CO_2 alone were dominated by lactic acid bacteria, principally *Leuconostoc* spp. and *Lactobacillus* spp. When N_2 and CO_2 were used together, along with lactic acid bacteria, *Bro. thermosphacta*, some coryneforms, and *Enterobacteriaceae* were also found. In the presence of some O_2, lactic acid bacteria, *Bro. thermosphacta, Enterobacteriaceae*, and *Pseudomonas* spp. were isolated. The product storage life, depending on a product, can be 4 weeks or more for fresh products and 8 weeks or more for processed products.[2–6]

VI. CONCLUSION

The use of modified atmosphere to reduce O–R potential of the environment has been a very widely used method to control growth mainly of aerobic microorganisms in food. However, facultative anaerobes and anaerobes can gain an advantage because of the absence of competition from aerobes. They can also be controlled in an O–R potential reduced food by combining other methods, especially some chemical preservatives, which are discussed in Chapter 37.

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QUESTIONS

1. Describe the methods used for the alteration of atmosphere to preserve foods.
2. Discuss the mechanisms of antimicrobial action in the MAP method used in foods.
3. Explain the different factors that can inhibit or influence microbial growth in a MAP food.
4. List the predominant microorganisms that can grow during the storage of vacuum-packaged and gas-flushed-packaged foods.
5. What is the major microbiological concern of MAP foods? How can this be solved?
6. *Lis. monocytogenes* has been implicated in foodborne disease outbreaks from the consumption of vacuum-packaged refrigerated low-heat-processed meat products. These products are also spoiled (gas fermentation) by *Leuconostoc* spp. Briefly discuss how these heat-sensitive bacterial species can be implicated in health hazard and spoilage in the properly heated packaged products. Also suggest a methodology to reduce the problems.

CHAPTER 37

Control by Antimicrobial Preservatives

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I. INTRODUCTION

Many chemical compounds, either present naturally, formed during processing, or legally added as ingredients, can kill microorganisms or control their growth in foods. They are, as a group, designated as antimicrobial inhibitors or preservatives.[1,2] Some of the naturally occurring preservatives can be present in sufficient amounts in foods to produce antimicrobial action, such as lysozyme in egg white and organic acids in citrus fruits. Similarly, some of the antimicrobials can be formed in enough quantities during food processing to control undesirable microbial growth, such as lactic acid in yogurt fermentation. However, others can be naturally present or formed in small quantities and essentially do not produce antimicrobial action in foods, e.g., lysozyme in milk (13 μg/100 ml) and diacetyl in some fermented dairy products. Among the many food additives, some are specifically used to preserve foods against microorganisms (such as NO_2 in cured meat to control spore germination, especially of *Clo. botulinum*) whereas others are added principally to improve the functional properties of a food (such as butylhydroxyanisol, BHA, used as an antioxidant, although it has antimicrobial properties). Use of some of the preservatives started as far back as 6000 B.C. Examples include salting (which had nitrate as a contaminant) of fish and meat; burning sulfur to generate SO_2 to sanitize the environment and equipment used for baking and brewing; smoking fish and meats; and adding spices, herbs, acids, and alcohol to foods (from fermentation).

Following the discovery of the microbial role in food spoilage and in foodborne diseases, many chemicals have been introduced since the late 1900s to control microorganisms in foods. The safety of the chemicals on humans was not tested before adding to foods, and some of the chemicals used were later found to be harmful to humans. To protect U.S. consumers, in 1958, the Food Additive Amendment was passed (see Appendix C). According to this amendment, a food processor wanting to use a food preservative and other additives must prove their safety, by the procedures recommended, before their incorporation. This law, however, granted an exemption to substances used for a long time, found to be safe, and considered as generally regarded as safe (GRAS) substances.

In recent years, the possible effect of different preservatives and other additives on human health from long-term use through different foods has been questioned. Many of them are of nonfood origin or added at a level not normally present in foods, and some are synthetic. The effect of interactions of the different preservatives in the body consumed through different foods, especially in children and debilitated and elderly people, the possible cumulative effect from the consumption for many years, and their interactions with each other and with other chemicals (e.g., medications) have not been determined. Many health-conscious consumers are interested in foods that do not contain any preservatives, especially those not normally found in foods or present in much a lower concentration than that added to foods. This has resulted in the search for preservatives that are either naturally present in the food of plant and animal origin or produced by safe food-grade microorganisms used to produce fermented foods. They are also designated as biopreservatives.[1] Some of these have been used, not always as preservatives, for a long time in foods and have been found to be safe (such as lactic, acetic, and propionic acids), whereas

others have not been used directly as preservatives. Although many of them have been unknowingly consumed through foods without any adverse effects (such as bacteriocins of many lactic acid bacteria), they probably have to be tested for safety and regulatory approval before use in foods.

II. OBJECTIVES

Antimicrobial chemicals are used in food in relatively small doses either to kill undesirable microorganisms or to prevent or retard their growth. They differ greatly in the abilities to act against different microorganisms (broad spectrum). Some are effective against many microorganisms, whereas others are effective against either molds and yeasts or only bacteria (narrow spectrum). Similarly, some compounds are effective against either Gram-positive or Gram-negative bacteria, or bacterial spores, or viruses. Those capable of killing microorganisms are designated as germicides (kill all types), fungicides, bactericides, sporicides, and viricides, depending on their specificity of killing actions against specific groups. Those that inhibit or retard microbial growth are classified as fungistatic or bacteriostatic. However, under the conditions in which most antimicrobials are used in foods, they cannot completely kill all the microorganisms or prevent their growth for a long time during storage. They can also cause injury.

III. INFLUENCING FACTORS

Several factors need to be considered in evaluating the suitability of an antimicrobial agent as a food preservative,[1–5] based on their antimicrobial properties, suitability for application in a food, and ability to meet regulatory requirements. As regards antimicrobial properties, a compound that kills (-cidal) instead of controlling growth (-static) is preferred. Similarly, a compound with a broader antimicrobial spectrum is more suitable for application in foods so that it is effective against many types of microorganisms important in foods (namely, molds, yeasts, bacteria, and viruses), as compared with one that has a narrow spectrum. Also, a compound effective not only against vegetative cells but also against spores is preferred. Finally, it should not allow development of resistant strains. Most compounds do not meet all these requirements. Many times, more than one compound is used in combination to increase the inhibitory spectrum. In addition, food environments may restrict growth of many types of microorganisms. Under such circumstances, a preservative that can effectively control the growth of the microorganisms of concern can be used alone.

To be suitable for application in a food, a compound should not only have the desired antimicrobial property but also not affect the normal quality of a food (texture, flavor, or color). It should not interact with food constituents and become inactive. It should have a high antimicrobial property at the pH, A_w, Eh, and storage temperature of the food. It should be stable during the storage life of the food. Finally, it should be economical and readily available.

The regulatory requirements include the expected effectiveness of an antimicrobial agent in a food system. It should be effective in small concentrations and should not hide any fault of a food (e.g., conceal poor quality and spoilage). Most importantly, it should be safe for human consumption. Finally, when required, it should be listed in the label, indicating its purpose in the food.

IV. EXAMPLES OF ANTIMICROBIAL PRESERVATIVES

Foods can have antimicrobial compounds in three ways: present naturally, formed during processing, or added as ingredients. Those added have to be GRAS-listed and approved by regulatory agencies. Some of these are added specifically as antimicrobial preservatives, whereas others, although having antimicrobial properties, are added for different reasons. Those in the latter group are also called indirect antimicrobials. In this chapter, the antimicrobial effectiveness, mode of action, and uses in foods of some inorganic and organic antimicrobials added to foods are briefly discussed.[1–7] Table 37.1 lists some antimicrobial preservatives (direct or indirect) used in foods.[1–6]

A. Nitrite ($NaNO_2$ and KNO_2)

Curing agents that contain nitrite, and together with NaCl, sugar, spices, ascorbate, and erythorbate, are permitted for use in heat-processed meat, poultry, and fish products to control growth and toxin production by *Clostridium botulinum*. Nitrate and nitrite are also used in several European countries in some cheeses to prevent gas blowing by *Clo. butyricum* and *Clo. tyrobutyricum*. The mechanisms of antibacterial action of nitrite are not properly understood, but the inhibitory effect is probably produced in several ways, such as reactions with some enzymes in vegetative cells and germinating spores, restriction of the bacterial use of iron, and interference with membrane permeability, thereby limiting transport. In addition to clostridial species, nitrite is inhibitory, to some extent, to *Staphylococcus aureus,*

Table 37.1 Some Antimicrobial Chemical Preservatives[a] Used in Foods

Acetaldehyde	Dehydroacetate	Lactic acid	Propylene glycol
Acetic acid	Diacetate	Lauric acid	Propylene oxide
Ascorbic acid	Diacetyl	Lysozyme	Propyl gallate
Bacteriocins	Diethyl dicarbonate	Malic acid	Smoke
Benomyl	Diphenyl	Methyl bromide	Sodium chloride
Benzoic acid	Ethyl alcohol	Monolaurin	Sorbic acid
Betapropiolactum	Ethyl formate	Natamycin	Spices
BHA, BHT, and TBHQ	EDTA	Nitrite and nitrate	Succinic acid
Boric acid	Ethylene oxide	Parabens	Sucrose
Caprylic acid	H_2O_2	Peracetate	Sulfites and SO_2
Chitosan		Polyphosphates	Tetracyclines
Citric acid		Propionic acid	Thiabendazole
CO_2 and CO			Tylosin

[a]Not all are permitted in the U. S.

Escherichia, Pseudomonas, and *Enterobacter* spp. at 200 ppm; *Lactobacillus* and *Salmonella* serovars seem to be resistant to this concentration of NO_2.

The antibacterial effect of NO_2 is enhanced at lower pH (pH 5.0 to 6.0), in the presence of reducing agents (e.g., ascorbate, erythorbate, and cysteine), and with sorbate. The current regulatory limit in the U.S. is 156 ppm of NO_2, but this varies widely in other countries. This amount can also be reduced by supplementing NO_2 with other reducing agents as well as sorbates. The NO_2 effect is also enhanced by reducing A_w and at low Eh. In cured meat products, NO_2 reacts with myoglobin to form a stable pink color of nitrosyl hemochrome during heating. In bacon, nitrite can lead to the formation of carcinogenic compounds, nitrosoamines. Because of this, there is a trend to reduce NO_2 or to use other preservatives to control *Clo. botulinum* in low-heat-processed meat products.

B. Sulfur Dioxide (SO_2) and Sulfites (SO_3)

Sulfur dioxide, sodium sulfite (Na_2SO_3), sodium bisulfide ($NaHSO_3$), and sodium metabisulfite ($Na_2S_2O_5$) are used to control microorganisms (and insects) in soft fruits, fruit juices, lemon juices, beverages, wines, sausages, pickles, and fresh shrimp.

Currently, these additives are not permitted in the U.S. in meat, as they destroy vitamin B1. They are more effective against molds and yeasts than bacteria; among bacteria, the aerobic Gram-negative rods are the most susceptible. The antimicrobial action is produced by the undissociated sulfurous acid that rapidly enters the cell and reacts with the thiol groups in structural proteins, enzymes, and cofactors, as well as with other cellular components. At low pH ($\leq$4.5) and low A_w, the fungicidal effect is more pronounced. In bacteria, they are effective at high pH ($\geq$5.0), but are probably bacteriostatic at lower concentrations and bactericidal at higher concentrations. The concentrations used in foods vary greatly in different countries. In the U.S., 200 to 300 ppm is generally permitted for antimicrobial uses.

Sulfur dioxide and sulfites are also used as antioxidants in fresh and dried fruits and vegetables (salads) to prevent browning. However, people with respiratory problems can be mildly to severely allergic to sulfites. The products need to be labeled to show the presence of sulfites.

C. H_2O_2

A solution of H_2O_2 (0.05 to 0.1%) is recommended as an antimicrobial agent in raw milk to be used in cheese processing (to control growth of psychrotrophic Gram-negatives that produce heat-stable enzymes), liquid egg to facilitate destruction of *Salmonella* by low-heat pasteurization, packaging material used in aseptic packaging of foods, and food processing equipment. In raw milk and liquid egg, catalase is used before pasteurization to hydrolyze H_2O_2 to water and oxygen. H_2O_2 is a strong oxidizing agent, and the germicidal action is associated with this property (see Chapters 16).

Recently, H_2O_2 has been used to produce modified plant fiber flour from straws for use in low-calorie foods and for bleaching and color improvement of grains,

chocolate, instant tea, fish, sausage casings, and many others, and to reduce sulfite in wines. In the future, the use of H_2O_2 in foods is expected to increase.

D. Epoxides (Ethylene Oxide, Propylene Oxide)

Ethylene oxide and propylene oxide are used as fumigants to destroy microorganisms (and insects) in grains, cocoa powder, gums, nuts, dried fruits, spices, and packaging materials. They are germicidal and effective against cells, spores, and viruses. Ethylene oxide is more effective. Epoxides are alkylating agents and react with various groups (e.g., –SH, $–NH_2$, and –OH) in cellular macromolecules, particularly structural proteins and enzymes, adversely affecting their functions. They can react with some food components, such as chlorides, and form toxic compounds that can remain as residue in treated foods. They can be toxic at high concentrations (as residue), particularly to people who are sensitive to them.

E. Acids

Acetic, propionic, lactic, citric, benzoic, and sorbic acids are discussed in Chapter 16 and Chapter 35.

F. Parabens

Methyl and propyl esters of *p*-hydroxybenzoic acids are discussed in Chapter 35.

G. Bacteriocins of Lactic Acid Bacteria

Bacteriocins of food-grade lactic acid bacteria, with special reference to nisin and pediocin AcH, are discussed in Chapter 16.

H. Diacetyl

Diacetyl is discussed in Chapter 16.

I. CO_2

CO_2 is discussed in Chapter 36.

J. Butylated Hydroxyanisol (BHA), Butylated Hydroxytoluene (BHT), and *t*-Butyl Hydroquinone (TBHQ)

BHA, BHT, and TBHQ are primarily used at 200 ppm or less as antioxidants to delay oxidation of unsaturated lipids. Additionally, they have antimicrobial properties and thus can be regarded as indirect antimicrobials. In concentrations of 50 to 400 ppm, BHA inhibits growth of many Gram-positive and Gram-negative bacteria; however, some species may be resistant to it. They also effectively prevent growth and toxin production by molds and growth of yeasts, but BHA seems to be more

effective. The antimicrobial action is most likely produced by their adverse effect on the cell membrane and enzymes. Their antimicrobial effectiveness increases in the presence of sorbate but decreases in foods with high lipids and at low temperature.

K. Chitosan

Chitosan, a polycationic polymer, is obtained by alkaline hydrolysis of chitin from the shells of Crustaceae. It has many applications in foods, including food preservation, because of its antimicrobial capability. It causes destabilization of the cell wall and cell membrane functions and is effective against bacteria, yeasts, and molds.

L. Ethylenediaminetetraacetate (EDTA)

The sodium and calcium salts of EDTA at 100 ppm are approved for use in foods to chelate trace metals in order to prevent their adverse effect on food quality. At a low dose (5000 ppm), EDTA appears to have no toxic effect and mostly passes through the GI tract unabsorbed. By itself, EDTA may not have much antimicrobial effect, but because of its ability to chelate divalent cations, it can destabilize the barrier functions of the outer membrane of Gram-negative bacteria and, to some extent, the cell wall of Gram-positive bacteria. In this way, it enhances antibacterial action of other chemicals, especially those that are membrane acting, such as surface-active compounds, antioxidants, lysozymes, and bacteriocins. EDTA is also inhibitory for germination and outgrowth of spores of *Clo. botulinum*. In the presence of divalent cations in the food environment (e.g., dairy products), the effectiveness of EDTA is greatly reduced.

M. Lysozyme

The enzyme lysozyme (a muramidase) is present in large quantities in some foods such as egg white and shellfish (oysters and clams), as well as in small amounts in milk and some plant tissues. It hydrolyzes the mucopeptide layer present in the cell wall of Gram-positive bacteria and in the middle membrane of Gram-negative bacteria. However, Gram-negative bacteria become sensitive to the lysozyme effect only after the barrier function of the outer membrane is destabilized by chemical (e.g., EDTA) and physical (e.g., freezing or heating) stresses. The antimicrobial effect is manifested by the lysis of cells. Lysozyme is most effective at pH 6.0 to 7.0 and at concentrations of ca. 0.01 to 0.1%. It can be used directly to control Gram-positive bacteria and with EDTA and other similar compounds to control Gram-negative bacteria. It has been used in wine (sake) to prevent growth of undesirable lactic acid bacteria.

N. Monolaurin (Glycerol Monolaurate)

Monolaurin, the ester of lauric acid and glycerol, is one of the more effective bactericidal agents among the different derivatives of lauric acid tested in foods. Its effectiveness in deboned meat, chicken sausages, minced fish, and other foods has been observed against undesirable bacteria, particularly the anaerobes. It also

enhances the thermal inactivation of spores of *Bacillus* spp. The antimicrobial property of this lipophilic compound is enhanced with lactate, sorbate, ascorbate, and nisin, but may be reduced by starchy and proteinaceous compounds. In combination with monolaurin, the fungistatic activity of several antifungal compounds is enhanced. The antimicrobial activity of monolaurin is produced through its ability to destabilize the functions of the membrane. At lower concentrations, it is bacteriostatic by interfering with the uptake of nutrients. It can be used up to 500 ppm without affecting the taste of the food.

O. Antibiotics (Tetracyclines, Natamycin, and Tylosin)

Several classical antibiotics that do not include bacteriocins of Gram-positive bacteria (such as nisin, pediocin, sakacin, and subtilin) were studied as antimicrobial food preservatives. Tetracyclines (ca. 10 ppm) were approved by the Food and Drug Administration (FDA) to extend the refrigerated shelf life of seafood and poultry in the 1950s. However, because of the possible increase in antibiotic-resistant bacteria, the use of these antibiotics in food was later banned. Natamycin, a microlid produced by *Streptomyces natalensis*, is an antifungal agent. Its use as a dip or spray to prevent growth of molds and formation of mycotoxins on the surface of some cheeses, sausages, and in raw peanuts has been approved by the Expert Committee of the World Health Organization (WHO). It is customarily used at 500 ppm, which leaves detectable but safe levels of the antibiotic on the product surface. Tylosin, a microlid that inhibits protein synthesis, is a bactericidal antibiotic that is more effective against Gram-positive than Gram-negative bacteria and also inhibits outgrowth of germinated endospores. Because of its high heat resistance, it has been studied at a low concentration (1 ppm) to determine its effectiveness in controlling the growth of sporeformers in low-acid canned products.

P. Wood Smoke

Many processed meat products and fishes are processed with smoke generated by burning hardwood, such as hickory, oak, maple, walnut, and mahogany. As an alternative, liquid smoke, obtained as a distillate of hardwood smoke, is also used with the ingredients of the products. The main reason for smoking meat, fish, and cheese is to impart desirable flavor, texture, and color to the products. The other benefit is the long shelf life of smoked products, especially those exposed to smoke during heating. The smoke contains several different types of chemicals that deposit on the food surface, many of which have antibacterial properties. The most important antibacterial agents are formaldehyde, phenols, and cresols. Depending on the temperature and time of heating, degree of surface drying (A_w), and the concentrations, smoking can be both bacteriostatic and bactericidal to bacterial cells. Although smoke has a slight antifungal action, it does not have any adverse effects on the survival or germination of bacterial spores. Liquid smoke, under similar conditions, is less antimicrobial than wood smoke. Smoke also contains some chemicals that are carcinogenic, such as benzopyrene and dibenzanthracene. One of the recommendations to reduce colon cancer is to minimize the consumption of foods treated with smoke.

Q. Spices

Many spices, condiments, and plant extracts are known to contain antimicrobial compounds. Some of these include cinnamic aldehyde in cinnamon; eugenol (2-methoxy-4-allyl phenol) in cloves, allspice, and cinnamon; and paramene and thymol in oregano and thyme. Their bacteriostatic and fungistatic properties depend on the active agent. Because of the small amounts used as spices in foods, they probably do not produce any antimicrobial effects. However, the antimicrobial components can be used in higher concentrations as oleoresins or essential oils.

The antimicrobial properties of garlic, onion, and ginger, as well as cabbage, Brussels sprouts, carrots, and others have generated interest for their possible use as natural preservatives. It is expected that in the future the antimicrobial compounds from plants, especially food plants, will be studied more effectively and thoroughly.

V. CONCLUSION

Many chemical compounds, approved by regulatory agencies, have been used to control growth of pathogenic and spoilage microorganisms in food. In addition, some of them can kill microbial cells and prevent germination of bacterial spores at concentrations used. Many of these compounds are more effective in combination with other methods. However food environments can greatly influence their effectiveness. They are, like the methods discussed before (except heat), not used to eliminate pathogens from a contaminated food. However, irradiation (besides heat) is used specifically to kill microbes present in food and is discussed in Chapter 37.

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QUESTIONS

1. Discuss the uses of antimicrobial chemicals before and after 1865 A.D., after 1958, and since the 1980s.
2. Read the labels of ten different foods from at least five food categories and identify in them the chemicals having antimicrobial action (direct or indirect).
3. Define the terms bacteriostatic, germicidal, bactericidal, fungicidal, sporicidal, viricidal, and sporostatic. Give one example of a preservative for each.
4. List five characteristics one should consider in selecting a suitable chemical preservative for a specific food. What is a GRAS-listed preservative?
5. Discuss the antimicrobial properties and any health concern of the following chemical preservatives, and list two foods (for each) in which they are used: NO_2, sulfites, bacteriocins, parabens, benzoic acid, lysozyme, natamycin, and lactic acid.
6. Indicate the mechanism of antimicrobial actions of a bacteriocin, lysozyme, and EDTA. From the information indicate how, if used together, they can produce a synergistic antimicrobial effect.

CHAPTER 38

Control by Irradiation

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I. INTRODUCTION

A. Irradiation (Radiation) and Radioactivity

In the electromagnetic spectrum, energy exists as waves and the intensity of the energy increases as the waves get shorter. On either side of visible rays (~400 to

800 nm) are invisible long waves (>800 nm; IR and radio waves for radio, TV, microwave, and radar) and invisible short waves (<300 nm; UV rays, x-rays, β-rays, γ-rays, and cosmic rays). Exposure to long waves, visible light waves, and UV rays does not cause any change in the atomic structures. In contrast, exposure to x-rays, β-rays, and γ-rays can remove electrons from the outer shell of an atom and thus form an ion pair (negatively charged and positively charged). Ion formation or ionization does not make an atom radioactive. To induce radioactivity, the nucleus of an atom has to be disrupted by much higher energies, such as by neutrons; x-rays, β-rays, and γ-rays do not have that much energy.

For application in food preservation, x-rays, β-rays, and γ-rays were studied for their ability to penetrate inside foods and kill microorganisms, their efficiency, and their effect on food quality. β-rays (actually electrons, similar to cathode rays) have very little penetration power; they cannot penetrate inside metal cans and are thought to be ineffective in food preservation. X-rays, although having good penetration power, cannot be effectively focused on foods and, because of this low efficiency, are not considered favorably for application in food. In contrast, γ-rays (photons) have high penetration power (~40 cm thick) and may be considered effective and economical for use in foods. Cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs) are considered to be good sources of γ-rays. ^{60}Co, an artificially induced radioactive isotope, is produced for use in nuclear medicine. When its energy level becomes so low that it cannot be further used in medicine, it is used to irradiate foods. Because of the easy availability of ^{60}Co over ^{137}Cs, it is preferred for food irradiation. Thus, foods irradiated with ^{60}Co do not become radioactive. Instead, the atoms (and molecules) in a food and in microbial cells form ion pairs and other components that inhibit the multiplication of microorganisms (and food cells, such as the inhibition of sprouting in potatoes).[1]

B. Use of Irradiation in Food

The ability of x-rays, γ-rays, and β-rays to kill microorganisms was recognized soon after their discovery in the late 19th and early 20th centuries. Some were even tested to determine their effectiveness in killing microorganisms in food in those early years. However, until the late 1940s and early 1950s, their use in food preservation was not actively studied. One reason was the unavailability of a large economical supply of radioisotopes, the technology of which was developed during World War II. Early studies showed that different foods can be irradiated to extend shelf life without making them radioactive, but the process affected the flavor qualities of the foods. Later, the problems were overcome by reducing exposure time and lowering product temperature. At the time, it was thought that irradiation of food would give storage stability for indefinite periods of time. The U.S. Army started a research program to determine whether storage-stable, acceptable, safe army rations could be produced by irradiation. Although foods do not become radioactive at low doses of irradiation, it was found that food accumulated radiolytic products. Extensive studies showed that they are present in very low concentrations (~3 ppm). Most of them are not unique to irradiated foods, because they are present naturally in different foods and also are produced in heated foods (thermolytic products). Well-designed

feeding studies, with animals as well as humans for a fairly long period, showed that irradiated foods did not cause any toxic effect or genetic defects. A WHO expert committee on the wholesomeness of irradiated food, after extensive review of more than 200 well-designed studies conducted worldwide, has recommended irradiation of food up to a certain dose level (10 kGy). This level is currently approved in more than 30 countries for use in meat, fish, vegetables, fruits, and grains. Many countries are regularly selling irradiated foods, especially some vegetables, fruits, and grains. However, consumer resistance has been quite high. This is mainly due to their lack of information and understanding that irradiated foods are not radioactive and radiolytic products are not unique to only irradiated foods. This is probably the only food preservation method that has been studied for safety for a long time (more than 40 years) before its recommendation, and whose use in many countries has been found to be safe by the world body of expert scientists. The other reason is political. A small but vocal group of lobbyists are waging a very successful campaign against the acceptance of irradiated food by large numbers of consumers. As a result, food industries are in a wait-and-watch situation.

There is no controversy among experts that food irradiation is an economical and effective food preservation method. As the world population increases and food production decreases, effective preservation methods must be used to feed the hungry mouths, otherwise social unrest may start. Politics and lobbying will not stop hunger, but food preservation methods (such as irradiation) will.

II. OBJECTIVES

A food is irradiated because of the destructive power of ionization on microorganisms a food harbors. Depending on the method used, it can either completely or partially destroy molds, yeasts, bacterial cells and spores, and viruses. In addition, irradiation can destroy worms, insects, and larvae in food. It also prevents sprouting of some foods, such as potatoes and onions. However, irradiation cannot destroy toxins or undesirable enzymes in a food, in which respect it differs from heat treatment (heat also does not destroy heat-stable toxins and enzymes). Irradiation is a cold sterilization process inasmuch as the temperature of a food does not increase during treatment, and thus irradiated foods do not show some of the damaging effects of heat on food quality. However, irradiation can cause oxidation of lipids and denaturation of food proteins, especially when used at higher doses.[1–3]

III. MECHANISMS OF ANTIMICROBIAL ACTION

When an object (food or microorganism) is exposed to high-energy γ-rays (10^{-1} to 10^{-2} nm), the energy is absorbed by thousands of atoms and molecules in a fraction of a second, which strips electrons from them. This produces negative and positive ion pairs. The released electrons can be highly energized and thus can remove electrons from other atoms and convert them into ions. This energization and ionization can adversely affect the normal characteristics of biological systems.

Ionizing radiation produces both direct and indirect effects on microorganisms. The direct effect is produced from the removal of electrons from the DNA, thereby damaging them. The direct effect is produced from the ionization of water molecules present in the cell. The hydrogen and hydroxyl radicals formed in this process are highly reactive and cause oxidation, reduction, and the breakdown of C–C bonds of other molecules, including DNA. Studies have shown that both single- and double-strand breaks in DNA at the sugar–phosphate backbone can be produced by the hydroxyl radical. In addition, the radicals can change the bases, such as thymine to dihydroxydihydrothymine. The consequence of these damages is the inability of microorganisms to replicate DNA and reproduce, resulting in death.

In addition to DNA damage, ionizing radiation also causes damage in the membrane and other structures, causing sublethal injury. Some microorganisms can repair the damage to the DNA strands (especially single-strand breaks) and in the bases, and are designated as radiation-resistant microorganisms.

Microbial death by ionizing radiation, as in heat treatment, occurs at a predictable rate, which, like heat, is dependent on dose (strength and exposure time), microbial species, and environmental factors. Because of this, the *D* value (minutes to reduce cell viability by 1 log of a species in a population at a given exposure) can be derived. This, in turn, can be used to determine the time necessary to reduce the population to a desirable level under a specific condition of treatment.

When microorganisms are exposed to UV radiation (≅260 nm), the energy is absorbed by the nucleotide bases in the DNA. The bases can react with each other to form dimers (e.g., thymine dimers) and cause breaks in the DNA strand. Microbial death and injury are associated mainly with DNA damage.[1–3]

IV. INFLUENCING FACTORS

A. Nature of Process

Among the several methods available (x-rays, β-rays, γ-rays), γ-rays have a higher potential for effective and economical use in food preservation. ^{60}Co is predominantly used in food irradiation because it is more readily and economically available. It has a half-life of ~5.3 years. It continuously emits γ-rays, and thus can be lost even when it is not used. ^{137}Cs can also be used in foods, but it is relatively difficult to obtain and is also required in larger amounts. The antimicrobial efficiency of ionizing radiation increases as the dose is increased. The antimicrobial efficiency, however, decreases in the absence of oxygen (because of reduced oxidizing reactions) and at low A_w (because of reduced free-radical formation with less water). Freezing also reduces the efficiency because of reduced availability of reactive water molecules.[1–3]

B. Nature of Foods

γ-Rays have a penetration capability of ca. 40 cm and can penetrate through paper, plastic, and cans. Thus, foods can be exposed to γ-radiation in packages, cans,

baskets, and bags. Frozen, dry, or anaerobically packaged foods need higher doses of treatment to obtain the desirable antimicrobial effect. In contrast, treatments such as curing, high hydrostatic pressure, high temperature, and low pH enhance the antimicrobial effect of radiation in food. Food compositions (thickness and particle size) also determine the efficiency of irradiation in reducing microbial numbers.

C. Nature of Microorganisms

Microorganisms vary greatly in their sensitivity to ionizing (and UV) radiation. Because of size differences, molds are more sensitive than yeasts, which are more sensitive than bacterial cells; bacterial cells are more sensitive than viruses (including phages). Among bacteria, Gram-negative cells are more sensitive than Gram-positive bacteria, and rods are more sensitive than cocci. Species and strains of bacterial cells vary greatly in their sensitivity to irradiation. Some strains, designated as radiation resistant, have effective metabolic systems to repair the cellular damages (especially single- and double-strand breaks of DNA and base damage). These include some bacterial strains that are important in foods, such as *Salmonella* Typhimurium*, Escherichia coli, Enterococcus faecalis,* and *Staphylococcus aureus.* Spores are quite resistant to irradiation, probably because their water content is very low. Among the sporeformers, spores of *Clostridium botulinum* Type A and *Bacillus pumilus* are probably the most resistant to irradiation. Generally, *Bacillus* spores (aerobes) are less resistant than *Clostridium* spores (anaerobes).

The rate of death of microorganisms by irradiation follows first-order kinetics (i.e., straight line patterns) as the thermal destruction curve and can thus be expressed as a *D* value (time to destroy 90% of viable microorganisms). Accordingly, it can be influenced by a higher initial population, relative numbers of resistant cells in the population, number of spores present, and age and condition of growth of a strain. Toxins of microorganisms are not destroyed by ionizing radiation at the dose levels recommended in foods. Although irradiation can cause mutations in some microbial cells in a population, either a possible increase in pathogenicity or an induction of a gene that transcribes for a toxin is not expected to occur, as observed from the many studies conducted in these areas.

V. METHODS

A. Doses

Radiation dose was originally designated as *rad*, and 1 rad was defined as the quantity of ionizing radiation that results in the absorption of 100 ergs of energy per gram of an irradiated material. The current unit is gray (Gy), and 1 Gy is equivalent to 100 rad. When 1 kg of food absorbs the energy of 1 joule (1 joule = 10^7 ergs), it has received a dose of 1 Gy. According to the international health and safety authorities, foods irradiated up to 10,000 Gy (10 kGy) are considered safe.

The relative sensitivity of microorganisms to irradiation dose is a function of their size and water content. Approximate lethal dose levels for insects and different

microorganisms have been suggested as follows: insects, ≤1 kGy; molds, yeasts, bacterial cells, 0.5 to 10 kGy; bacterial spores, 10 to 50 kGy; viruses, 10 to 200 kGy. Thus, at the recommended level of 10 kGy, *Clo. botulinum* spores are not destroyed in foods (they need ca. 30 to 60 kGy), although cells of pathogenic (and spoilage) bacteria are destroyed. The products thus treated should have other barriers (low pH, low A_w, temperature ≤ 4°C) to control germination and growth of sporeformers. But some sporeformers (such as *Clo. laramie*) can germinate and multiply below 4°C. At present, a low dose level (<1 kGy) is used to control insects in fruits and grains, parasites in meat and fish, and sprouting in vegetables. Medium doses (1 to 10 kGy) are used to control foodborne pathogens and spoilage microorganisms to extend safety and stability of refrigerated foods. Higher doses (>10 kGy) to destroy spores are not used in foods except in spices and vegetable seasonings that are used in very small quantities.

Irradiated foods, such as postheat contamination in heated foods, can be contaminated later with pathogenic and spoilage microorganisms from various sources unless proper precautions (such as packaging) are used.[1–4] Also, a food can be reirradiated and not be detected.

B. Specific Terms

1. Radurization

Radiation pasteurization is mainly intended to destroy spoilage bacteria in high-pH–high-A_w foods, especially Gram-negative psychrotrophs in meat and fish and yeasts and molds in low-pH–low-A_w foods. The treatment is generally milder (≅1 kGy). The products should be packaged and chilled to prevent growth of pathogens, which were previously thought to be mesophiles. However, with the recognition of psychrotrophic pathogens and the importance of psychrotrophic Gram-positive spoilage bacteria, this treatment may not be effective.

2. Radicidation

This is the radiation of foods to destroy vegetative foodborne pathogens. The dose level used is ca. 2.5 kGy to 5.0 kGy. Although it is effective against pathogenic vegetative bacterial cells and molds, spores of the pathogens are not destroyed. Also, some radiation-resistant strains of pathogens can survive, such as some *Salmonella* Typhimurium strains. Irradiated products thus need to be stored under ≤4°C, especially to prevent germination and outgrowth of spores of *Clo. botulinum*.

3. Radappertization

This method involves radiation of food at a high dose (≅30 kGy) to destroy *Clo. botulinum* spores in order to get a safety similar to that by 12*D* heat treatment. However, this is not recommended for use in food.

C. Current Recommendations

Irradiated fresh fruits, vegetables, meat, and fish have been approved in 37 countries and are used in such nations as France, Belgium, and the Netherlands. In the U.S., irradiated foods have been used by the army and in the space program for some time. Before 1985, irradiation was permitted by regulatory agencies for spices, wheat, wheat flour (to destroy insects), and potatoes (to prevent sprouting). Subsequently, permission for irradiation was approved in 1985 for pork (against trichina), in 1986 for fresh foods (fruits and vegetables to destroy insects and larvae), and in 1992 for poultry and poultry parts (to destroy pathogens, specifically *Salmonella*). Currently, radiation of seafood (to destroy pathogens and spoilage bacteria) is being considered. In addition, in the U.S., radiation sterilization of beef steaks in the space program and refrigerated shelf-stable food for the military are being studied.

Currently, irradiated fruits (strawberry, mango, and papaya) and poultry are being marketed in limited amounts, and the consumer response has been favorable. Irradiated foods will have a special logo (Figure 38.1) along with the words "Treated with Radiation" or "Treated by Irradiation," for consumer information.

D. UV Radiation

Microorganisms are especially susceptible to UV light between 200 and 280 nm. Because of low penetration power, it has been used to inactivate microorganisms on the surface of foods (meat, fish, and bread) as well as in air and on walls, shelves, and equipment in the food handling and processing area. In addition, liquids, such as water and syrups, in thin layers have been treated with UV.

VI. CONCLUSION

Irradiation, like heat, kills microbial cells and destroys their spores at a predictable rate that is basically dependent on dose level, exposure time, and microbial type. Its effectiveness on microbial control in food has been studied well since the beginning



Figure 38.1 Logo for irradiated foods. The Codex Alimentarius, an international committee on food safety, has developed this logo to put in green color on the package of irradiated foods. Irradiated foods from the U.S. are labeled with this logo, along with the words "Treated with Radiation" or "Treated by Irradiation."

of the 20th century. Although irradiated foods have been found to be safe, many consumers view them as unsafe. As a result, irradiation is used in a limited way in food. It is expected that with proper education of consumers on the safety of irradiated food, consumers' demand will increase and irradiation will be used to destroy many foodborne pathogens in raw and processed foods. In recent years, several other methods that can be used to kill microorganisms effectively have been studied and are discussed in Chapter 39.

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QUESTIONS

1. Briefly discuss why irradiated foods are not commercially successful in many countries.
2. Explain the mechanisms of antimicrobial actions of irradiation by γ-ray and UV light.
3. List the factors that can reduce or increase the antimicrobial effectiveness of irradiation in food.
4. What recommended levels of γ-irradiation are approved in different foods and what are their objectives? What is the status of handling spore problems in irradiated foods?
5. Briefly explain the importance of the following in relation to food irradiation: (a) irradiated products, (b) radioactive products, (c) radiolytic products, (d) thermolytic products, (e) free radicals, (f) radarization, (g) radicidation, and (h) radappertization.
6. In a beef-processing operation, fresh beef in 30- to 50-lb sizes was vacuum packaged and effectively irradiated to destroy pathogenic bacteria. In a retail operation, the packages were then opened and cut into retail size (≅ to 1 lb). Some of these were found to contain pathogens. Discuss how this contamination could have occurred and suggest a method to overcome this problem.

CHAPTER 39

Control by Novel Processing Technologies

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I. INTRODUCTION

Many of the conventional food preservation methods used at present and discussed here have several disadvantages. High-heat treatment given to foods for safety and long shelf life results in loss of heat-sensitive nutrients (e.g., thiamine, riboflavin, folic acid, and vitamin C); denatures proteins and causes changes in texture, color, and flavor; and induces formation of new compounds through covalent bondings (e.g., lysinoalanine). Low-heat processing, such as pasteurization, minimizes the disadvantages of high-heat processing of foods, but the foods have limited shelf life even at refrigerated storage. Drying and freezing also reduce nutritional and acceptance qualities of food, especially when stored for a long time. Irradiated foods have not been well accepted by consumers. Many of the chemical preservatives used are of nonfood origin and have limited efficiency.

Since the 1980s, health-conscious consumers, especially in the developed countries, have been concerned about the possible adverse effects that "harshly produced" and "harshly preserved" foods might have on their health and on the health of future generations. There is a concern that the cumulative effects and interactions of different types of food preservatives on the human body during one's lifetime are not properly understood. The revelation about the harmful effects of some of the additives that were once allowed to be incorporated in foods has shattered consumer confidence. The philosophy of the consumers has changed from "How long will I live?" to "How well will I live?" which, in turn, has shifted the desire of these consumers to nutritious, natural, and minimally processed foods that have not been subjected to harsh processing or harsh preservation techniques. Because of changes in the socioeconomic patterns and lifestyles, many consumers are also interested in foods that have a long shelf life and take very little time to prepare.[1]

The suitability of several thermal and nonthermal processing and preservation methods are being studied to produce such foods. The principal advantages, disadvantages, and the current status of some of these methods are briefly described here. Major emphasis is given to their antimicrobial properties.[2,3]

II. SUMMARY OF PROCESSING METHODS

Some of the novel technologies currently being studied to process foods to control microorganisms as well as to produce new types of foods and their current status in the application of food are briefly described. This includes processing under thermal and nonthermal conditions. For further information, a recent review may be consulted.[3]

A. Microwave and Radio-Frequency Processing[3]

The methods involve exposing a food to electromagnetic waves at microwave and radio frequencies. Generally, microwaves at 915 MHz and 2450 MHz and radio frequencies at 13.6, 27.1, and 40.7 MHz are used in food applications, but very little study has been conducted with radio frequency. Both techniques generate internal

heat by dielectric and ionic mechanisms; water molecules, because of their dipolar nature, are associated with dielectric heating. Heating of food is very rapid but not uniform as compared with that in conventional heating. Destruction of microorganisms (cells and spores) is accomplished by the heat mainly through denaturation of proteins and nucleic acids. Microwave ovens are widely used now to cook and reheat foods. This method has the potential of producing commercially pasteurized and sterilized foods. However, because of the possible presence of cold spots in solid and semisolid foods, the method cannot be used to assure safety and is thus currently not regulated by the FDA (see Chapter 32). Many studies are now being conducted to improve the uniform heating of the microwave oven.

B. Ohmic and Inductive Heating[3]

In ohmic heating, electric currents are passed to heat food through an electrode contacting the food. In contrast, in inductive heating, electric coils placed near a food generate an oscillating electromagnetic field that sends electric currents to heat the food. Of the two, ohmic heating has been studied well for it potential use in the production of commercially pasteurized and sterilized food products. The destruction of microbial cells and spores is achieved primarily by destabilizing proteins, nucleic acids, membrane, and other vital functional components by heat. Food materials are heated rapidly and uniformly from the generation of internal energy. This method also produces cold spots in foods containing high fat and solids. Techniques are being studied to minimize their occurrence.

C. Pulsed Electric Fields[3,4]

Use of pulsed electric fields (PEF), a nonthermal method, involves application of high-intensity electric pulses between two electrodes placed in a food for a very short time to inactivate microorganisms. Various aspects of this method, including applications in food, have been studied. A short review of the method is presented in Section III of this chapter.

D. High-Pressure Processing[3]

High-pressure processing (HPP) is a nonthermal method involving pressurization of a packaged food in a water-filled closed chamber for a short duration to inactivate microorganisms. A liquid food can be pressurized directly. In addition to antimicrobial effect, it has many other food applications. The method has generated a great interest and several foods have already been commercially produced. A review of the current status of the method has been presented in Section IV of this chapter.

E. Pulsed Light Technology[3]

This is a nonthermal method in which microorganisms on the surface of food (usually in a transparent package) are inactivated by high-energy light pulses. Light pulses in the wavelengths of 170 to 2600 nm are used for a very short duration (≤0.01 s).

Processing units have been developed in which electric energy can be stored in a capacitor over a long period, which then can be released in a shorter time to produce an antimicrobial effect. The antimicrobial effect is produced through the action on nucleic acids (especially in the UV range), proteins, membrane, and other cellular components. The antimicrobial effectiveness of the process has been studied on food contact surface, packaging materials and surface of various foods, including cheeses, processed meat products, bakery products, and fishery products. Following treatment, the products retained acceptance qualities during extended shelf life.

F. Oscillating Magnetic Fields[3,5]

In this nonthermal method, a food, sealed in a plastic bag, is exposed to oscillating magnetic fields (OMF) (<500 kHz) for a duration to receive 1 to 100 pulses over a short period (≤100 ms). Limited studies showed that microbial inactivation is two to three log cycles, but the results were inconsistent. Some studies have been conducted using milk, yogurt, orange juice, and dough. The antimicrobial effect is produced from the damages in the DNA during exposure to the magnetic field.

G. Ultrasound[3]

Lysis of microbial cells by sonication forms the basis of the use of ultrasound to inactivate microorganisms in foods. The method consists of generating high-frequency sound waves in the test materials. The antimicrobial effect of ultrasound is attributed to intracellular cavitation that disrupts the cellular structures and functional components. Limited studies revealed that the antimicrobial effect of ultrasound in food is rather low. However, it can be enhanced by combining ultrasound with heat above 50°C. In addition to the antimicrobial effect, ultrasound has been studied as a means to determine texture, viscosity, thickness, and composition of different foods.

H. High-Voltage Arc Discharge[3]

In this method, stored high-voltage electrical energy is rapidly discharged in the form of an arc through the gap of electrodes placed in a liquid food. The process not only raises the temperature of the liquid to some extent but also produces electrohydraulic shock. Antimicrobial effect is produced mainly because of membrane damage by the electrohydraulic shock. The major disadvantages of the method are the generation of electrolytic products and disintegration of food particles. Improved techniques are now being developed to overcome the problems.

I. Pulsed x-Rays[3]

Irradiation of food by γ-rays using ^{60}CO (and ^{137}Cs) has been discussed in Chapter 38. One of the disadvantages of this method is that the radionuclides emit radiation in all directions and all the time; it is thus uneconomical and also hazardous. In contrast, x-rays can be generated only when they are needed and in the linear

direction they are needed. Pulsed x-ray, which delivers high-energy pulses in a very short time, is being studied to inactivate microorganisms in foods. The equipment can produce high energy with high penetrating power and least variation in dose uniformity. Limited studies have revealed that when used properly, the method can destroy five to six log cycles of microbial cells and spores at 20°C. The microbial inactivation occurs, like in γ-radiation, from the damages in the DNA.

III. PULSED ELECTRIC FIELD

The antimicrobial effect of high electric field pulses is not due to the electric heat or electrolytic products, but rather to the ability to cause damage to the cell membrane. When microbial cells in a suspension are exposed to pulses of high-voltage electric fields, a potential difference occurs between the outside and inside of the membrane. When the external electric field strength is moderately higher, so that the transmembrane potential does not exceed the critical value by 1 V, pore formation occurs in the membrane; the process is reversible (This principle is used in electroporation of cells to introduce foreign DNA.) However, if a much higher external electric field strength is applied so that membrane potential exceeds the critical value, the pore formation becomes irreversible, causing the destruction of membrane functions and cell death. For destruction of microbial cells, an electric field strength of ca. 15 to 25 kV/cm for 2 to 20 μs is necessary. Destruction of bacterial and fungal spores requires a higher voltage and a longer period of time.[3,4]

The lethal effect of PEF against microorganisms has generated interest for its use in nonthermal pasteurization and commercial sterilization of foods. During the process, there is little increase in the temperature of the suspension. However, to obtain a greater microbial destruction, the temperature of the suspension can be increased to 60ºC or higher. Also, by increasing the number of pulses, greater microbial destruction can be achieved.

In the PEF processing of food, a high-voltage pulse (20 to 80 kV/cm) is discharged between two electrodes in the food in a short time from a series of capacitors that acts as a storage. PEF can be applied as an exponentially decaying or square wave; the square wave appears to be more uniform and lethal. Pulses can be given once or more than once to enhance lethality. PEF treatment in the presence of other antimicrobial compounds (such as bacteriocins), at higher temperature (≥50°C), and lower pH is more lethal to microbes. A process called Elsterile has been developed in Germany for microbial destruction by PEF in liquid food. The liquid food, in a treatment chamber that has two carbon electrodes, is subjected to high-voltage electric pulses. A 4-log reduction was obtained for *Lactobacillus brevis* in milk by treating with 20 pulses at 20 kV/cm for 20 μs. A similar reduction was also observed by treating *Saccharomyces cerevisiae* in orange juice with five pulses at 4.7 kV/cm for 20 μs. The increased reduction of the yeast cells, as compared with bacterial cells, was thought to be due to the low pH of orange juice and larger cell size of yeasts.

In the U.S., the antimicrobial efficiency of PEF has been studied in several fluid foods, such as dairy products, fruit juices, and liquid egg products. Apple juice

treated with 10 pulses of PEF at 50 kV/cm for 2 μs each at 45°C retained its acceptance qualities and vitamins for 28 d at 4°C (compared with 21 d of fresh juice). Aerobic plate counts (APCs/ml) in orange juice were reduced by three to four log cycles by applying 32 kV/cm at ambient temperature, and the product had a 90-d shelf life at 4°C. The population of *Listeria innocua* was reduced by 2.5 log cycles in skim milk by PEF treatment at 50 kV/cm. PEF treatment in the presence of nisin reduced the *Listeria* population by 3.4 log. *Escherichia coli* and *Lis. monocytogenes* populations in milk were reduced respectively by 3 log and 2.5 log at ambient temperature. In liquid egg treated at 36 kV/cm at ambient temperature, APCs reduced by 2.7 log and the product had a longer shelf life than untreated products. Inactivation of microbial cells by PEF in particulate food has been studied. However, to achieve a comparable reduction (as with liquid foods), a much higher voltage was necessary.

Limited studies showed that at ambient temperature, PEF treatment had very little lethal effect on spores. However, in a combination of higher temperature (≅70 to 80°C), low pH, and the presence of EDTA and lysozyme, some spore inactivation can be achieved. A combination of 80°C heat shock, lysozyme, followed by PEF at 60°C reduced spores of a *Bacillus subtilis* strain by three to four log cycles.

These studies showed that PEF as a nonthermal processing technique can be used in liquid food to reduce mainly vegetative cells of spoilage and pathogenic microorganisms to extend its shelf life and ensure safety.

IV. HYDROSTATIC PRESSURE PROCESSING

A. History

Microbial cells exposed to high hydrostatic pressure (100 to 800 MPa or more) in a water-filled (or a suitable liquid-filled) closed chamber die rapidly. Bob Hite in 1890 first studied the effectiveness of destroying microorganisms by high hydrostatic pressure to preserve foods. But it took almost 90 years to apply high hydrostatic pressure to food preservation and production of several commercial foods in Japan. The technique, since then, has generated worldwide interest and has been referred to as ultrahigh hydrostatic pressure processing (UHP), high hydrostatic pressure processing (HHP), or simply hydrostatic pressure processing (HPP). Early studies were conducted with high-acid and low-protein fruit products, such as fruit juices, jams, and jellies, where the objective was to destroy aciduric spoilage microorganisms, mainly yeasts, molds, and lactic acid bacteria, as well as vegetative cells of some potent pathogenic bacteria. Some acid-sensitive vegetative bacteria and spores of spoilage and pathogenic bacteria were not considered because the cells do not multiply and spores do not germinate and outgrow in a low-pH (<4.6) environment. The current interest in HPP has included both low-pH (<4.6; high acid) and high-pH (≥4.6; low acid) foods and foods with high protein contents. At present, destruction of different foodborne spoilage and pathogenic bacteria, bacterial spores, yeasts, and molds is being studied. It will also be important to study the destruction of foodborne pathogenic enteric viruses, protozoa, and other parasites, as well as

bacteriophages of lactic acid bacteria. As HPP alone cannot destroy all the important microorganisms in different types of foods, parameters are being developed for specific food groups based on similarities in their intrinsic characteristics. The effectiveness of other parameters, such as pressurization time, pressurization temperature, presence of one or more antimicrobials during pressurization, pH, and A_w are also being studied. Like in heat processing, studies on pressure processing are directed toward pressure pasteurization to destroy vegetative cells of foodborne spoilage and pathogenic microorganisms and pressure sterilization to destroy foodborne bacterial spores. Whereas the pasteurization process can be nonthermal, the sterilization process is thermal. [Currently, the unit megapascal (MPa) is used to indicate pressure processing. The relationship between several units is as follows: 1 atm = 1 bar = 14.5 psi = 750 torr = 100 kilopascal (kPa) = 0.1 MPa].[3,6]

B. Methods, Mechanisms of Microbial Inactivation, and Advantages[3,6–10]

Hydrostatic pressure processing involves exposing a packaged solid or liquid food in water or water containing a little oil or an unpackaged liquid food (e.g., orange juice) in a closed steel chamber and then applying pressure by pumping more of the same liquid in the closed chamber (Figure 39.1). The pressure is transmitted instantaneously and uniformly throughout the closed chamber (as opposed to heat transmission). In general, the come up and come down times of pressure are very short (1 to 5 min). The pressurization causes the volume of the pressurized material to be compressed (10 to 15% between the range of 300 to 700 MPa) and a rise in temperature due to adiabatic heating (≅3°C/100 MPa). These changes are temporary and during depressurization they return to original states; however, both influence microbial inactivation, causing the phase transition of lipid bilayers and high-pressure disruption of ionic bonds, hydrophobic interactions, and hydrogen bonds of the molecules (without affecting the covalent bonds). This causes the macromolecules to unfold. When the pressure is released the molecules refold, but mostly in a manner different from the original configuration, changing the original characteristics. Volume compression (that brings the molecules closer) and adiabatic heating (due to temperature-related actions) augment these molecular changes. Among the molecules, most changes occur in the secondary and tertiary structures of proteins as well as association of proteins with other molecules. In microbial cells, many vital structural and functional components are adversely affected, causing viability loss as well as reversible or sublethal injury. Although damage to the cell membrane is viewed as the major cause of cell death, damages in cell wall, DNA, and RNA; inactivation as well as activation of some enzymes; and lysis of cells have been reported. These changes are enhanced by increasing the pressure, pressurization temperature, and pressurization time. Bacterial spores at a very high pressure (≥700 MPa) are probably inactivated by the inactivation of enzymes associated with the germination process. At a lower pressure range (≅200 to 300 MPa), spores are induced to germination by a mechanism similar to heat-shock germination. Once germinated, the outgrowing spores are susceptible to pressurization like the vegetative cells.

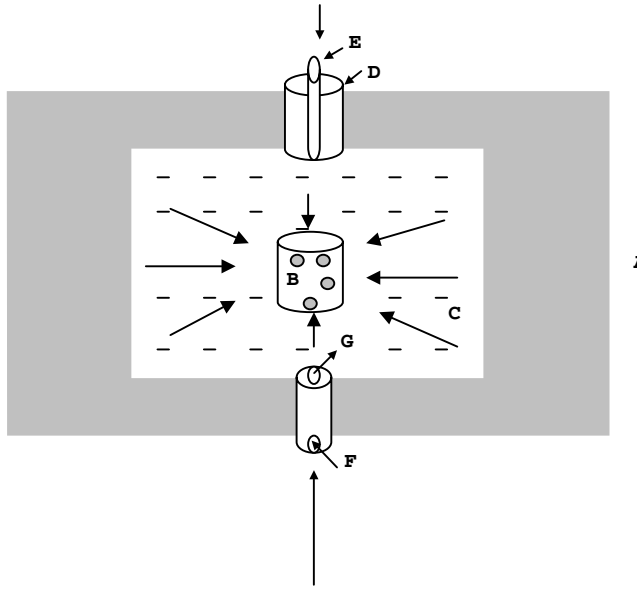


Figure 39.1 Schematic diagram of a hydrostatic pressure vessel (A). The packaged food (B) is put in the liquid from the top inside the chamber (C) and the top is closed by the movable cylinder (D) that has an opening valve (E) to removed air and excess liquid before pressurization. Once the chamber is closed, excess liquid is pumped in through the tube (F) that has a valve (G) that closes when the desired pressure is reached. The pressure is maintained constant during pressurization time, at the end of which the inlet valve (G) opens and the excess liquid is removed by gravity to bring the pressure inside the chamber to atmospheric pressure. Then the top cylinder is moved and the package is removed from the chamber. The liquid in the chamber can be heated to the desired temperature by installing a heating coil around the vessel (A). The temperature of the liquid inside the chamber can be monitored by placing a thermocouple in the top movable cylinder (D). The process is clean, energy efficient, and requires relatively less space to operate compared with other food-processing methods.

Hydrostatic pressure processing of food requires a very short time, less energy, and less space, and is safer than thermal processing. It does not act on covalent bonds of molecules; thus, vitamins, color pigments, and other small molecules are not adversely affected. Although it is basically a batch process, because of the short time requirements, it can be made continuous by installing three or more units in which while one is being filled, the second one is pressurized or depressurized and the third one is emptied. With the availability of units that can handle larger volumes, this becomes a commercial reality. The process has three parameters: pressure, temperature of pressurization, and time of pressurization. By adding suitable antimicrobials during pressurization, it can be made a four-dimensional unique process.

C. Destruction of Microbial Cells[3,6–10]

1. Bacterial Cells

There have been many studies on the pressure-induced viability loss and sublethal injury of foodborne bacterial cells, with more studies being reported for the pathogens. Some of these include pathogens such as *Salmonella* serovars, *Lis. monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Vibrio parahaemolyticus,* and *Esc. coli* O157:H7, and spoilage bacteria such as *Pseudomonas* spp., *Serratia liquefaciens, Leuconostoc mesenteroides,* and *Lactobacillus sake.* The antibacterial effect of hydrostatic pressure can be summarized as follows:

- Gram-negative bacteria are relatively more susceptible than Gram-positive bacteria and rods are more sensitive than cocci.
- Species and strains of a species differ in sensitivity.
- A strain can be sensitive to thermal treatment but resistant to pressure treatment and vice versa.
- Pressure resistance increases with higher consistency, lower A_w, higher pH, and higher lipid content of the suspending media.
- Viability loss increases directly with the increase in pressure, temperature, and time, with time having the least effect.
- Pressurization also induces sublethal injury in viable cells.
- Suitable antimicrobial agents (physical and chemical) can be included during pressurization to enhance viability loss.

Bacterial cell death in a given set of pressurization parameters occurs in a predictable way. Like in thermal treatment, the death rate follows first-order kinetics, especially at a pressure above 300 MPa and a pressurization temperature of 45°C and above. *D* values of eight different foodborne bacterial cell suspensions in peptone solution following pressurization at 345 MPa and 50°C were found to <0.6 min. Thus, by a suitable combination of pressure and temperature, a 5- to 6-log reduction can be achieved within 5 min; it is increased further by 1 to 3 log by incorporating an antimicrobial agent (e.g., a bacteriocin) during pressurization.

2. Bacterial Endospores

Bacterial spores are fairly resistant to pressures below 700 MPa at ambient temperature. However, pressures above 700 MPa at temperature above 70°C can be detrimental to them. The information available from reported studies can be generalized as follows:

- Spore inactivation is directly proportional to the levels of pressurization (at ≥ 700 MPa), pressurization temperature (at ≥ 70°C), and pressurization time (at ≥ 30 min).
- Spores of species and strains of species vary greatly in pressure resistance.
- In general, *Bacillus* spores are less resistant than *Clostridium* spores to pressure treatment.
- Dormant and superdormant spores are more pressure resistant.

- High concentrations of nonionic solutes and ionic solutes, low A_w, and high pH increase resistance.
- At a low pressure range (≅300 MPa), some spores germinate and outgrow, and can be killed by one or more physical and chemical antimicrobial agents.

Although in low-pH foods inactivation of spores is not necessary, it is necessary to destroy (or prevent germination and growth) foodborne *Bacillus* and *Clostridium* spores in high-pH foods. Most results indicate that even at 800 MPa and a pressurization temperature of 60 to 90°C, a 5-log cycle inactivation is difficult to attain in 10 min. Similarly, inducing germination of spores at a lower pressure range and then killing the germinated spores with a second cycle of pressurization does not ensure a predictable reduction of spores to achieve commercially sterile foods. It might be necessary to destroy some spores by a combination of parameters applicable for a food type and then prevent growth of the surviving spores with a suitable antimicrobial agent. This method is used in low-heat-processed meat and other food products. A recent study has reported that a combination of high pressure and high temperature and a second cycle of repressurization can be used to produce commercially sterile food. This is discussed later.

3. Molds, Yeasts, Viruses, and Parasites

Initial studies were conducted to develop pressurization parameters at ambient temperature to destroy yeasts and molds because of their ability to grow in low pH, low A_w, high osmotic environment, and refrigeration temperature in fruit juices, jams, and jellies. In general, a 4- to 5-log cycle of most foodborne yeasts and molds (including their spores) can be achieved by pressurizing at 300 to 350 MPa for 10 min at ambient temperature. Although a few pressure-resistant species and strains of these microbes have been found, treatment at 400 MPa at 40°C or a second cycle of pressurization eliminates them.

Almost no report is currently available on the pressure-induced destruction of foodborne protozoa and parasites in food systems. Similarly, the effect of pressure on the inactivation of foodborne pathogenic human viruses and bacteriophages of lactic acid bacteria (and prions) is not known. The current opinion is that the protozoa, parasites, and viruses will be destroyed by pressure at 300 to 400 MPa at ambient temperature. However, it is necessary to validate this by research.

D. Application in Food Processing[3,6–10]

Hydrostatic pressure is being studied for many applications in food processing, including killing microorganisms. Applications can be divided into three groups: novel product development, improving processing techniques, and antimicrobial applications, as listed in Table 39.1. Antimicrobial applications to preserve foods are briefly discussed here. Books and reviews may be consulted for information on other applications.[3,8]

Antimicrobial applications have been studied to produce mostly pressure-pasteurization products, although limited studies are now being conducted to produce

Table 39.1 Some Potential Applications of High Hydrostatic Pressure in Food

1. Antimicrobial applications
To destroy microbial cells at lower pressure range
To destroy bacterial spores at higher pressure range
To germinate bacterial spores at lower pressure range
To prevent microbial growth in unfrozen food
2. Novel product development
To produce fruit and vegetable products with natural taste
To improve color of fruit products and egg yolk
To facilitate gelation of proteins without heat
3. Improved food processing
To tenderize meat
To enhance cheese ripening
To open valves of shellfish
To inactivate undesirable food enzymes
To thaw frozen food rapidly
To store food unfrozen at –20°C
To inactivate allergens and toxins

commercially sterile products. Pressure pasteurization is considered nonthermal because the foods are pressurized (≤700 MPa) at a lower temperature range (15° to <70°C) to mainly kill vegetative cells of microorganisms, whereas pressure sterilization involves pressurization (≥700 MPa) at high temperature (≥90°C) to destroy bacterial spores. Many types of food are currently being studied for pressure pasteurization and a few are now commercially produced. A brief list of these is included in Table 39.2.

Some of the results of our studies with processed meat products at the University of Wyoming are presented here.[11] In each of these studies, a five-strain mixture of pressure-resistant *Lis. monocytogenes*, *Salmonella* serovars, and *Esc. coli* O157:H7 was inoculated either at a high ($\cong 10^7$/g) or a low ($\cong 10^3$/g) level to frankfurters in packages, some of which were then inoculated with a mixture of two bacteriocins, pediocin and nisin. The bags were then pressurized at 345 MPa for 5 min at 50°C. Packages with high-level inoculation were enumerated for the survivors immediately after pressurization. Packages with low-level inoculation were stored at 4°C, and at selected intervals, multiple packages were tested for the survivors both by enumeration and by isolation techniques. Results of high-level inoculation studies showed that pressurization in the presence of bacteriocin mixture is more lethal than pressurization alone (Table 39.3). The results of low-level inoculation studies revealed that by the enumeration technique alone, a package might indicate an apparent

Table 39.2 Current Status of Food Product Development

• Fruit products: juices, jellies, jams, cut fruits, fruit yogurt
• Vegetable products: avocado dip, salsa
• Meat products: roast beef, salami, sausage, frankfurters, foie gras
• Seafood products: smoked salmon cream, oysters, surimi
• Miscellaneous: Spanish rice, spaghetti, rice wine

Table 39.3 Viability Loss Determination of *Lis. monocytogenes* Strains by Pressurization in Inoculated Frankfurter (High-Level Inoculation)

Treatment[a]	Log CFU/g	Log Viability Loss/g
Control	7.6	—
PN	6.1	1.5
HP	3.4	4.2
HP + PN	ND[b]	~7.6

[a]Cell suspension of a five-strain mixture of pressure-resistant *Lis. monocytogenes* was inoculated in hotdog (sterile) packages (50g/package at 7.6 log CFU/g). Some of the packages were inoculated with a mixture of pediocin AcH and nisin (PN; 1:1; 3000 AU/g). Packages were pressurized at 345 MPa for 5 min at 50°C. Enumeration was done by surface plating on prepoured plates of tryptic soy agar. Each result is the average of four packages. Control, without any treatment; PN, with pediocin and nisin only; HP, only pressurized; HP + PN, pressurized in the presence of PN.
[b]No CFU was detected in 0.4 g sample/package.

absence of the pathogen because the technique cannot detect a very low level of survivors (theoretically ≅1 CFU/package). However, even from very few survivors in the package, a psychrotrophic pathogen can multiply during refrigerated storage to reach a detectable level (Table 39.4). In contrast, the isolation technique can be effectively used to detect any survivor in a package. Thus, for a highly potent pathogen for which a zero tolerance is desired, the processing parameters should be evaluated by the isolation technique. The results also showed that pressurization in the presence of a suitable antimicrobial (such as a bacteriocin mixture) is more effective in reducing foodborne bacteria.

Recently, an effective pulse pressurization technique has been developed to produce sterile foods.[12] In this, a food inoculated with the spores of *Bacillus* and *Clostridium* spp. is preheated to 90°C and given a first pressurization treatment at 690 MPa for 1 min; following depressurization and a pause for 1 min, it is pressurized again for 1 min at 690 MPa. This technique has produced sterile macaroni with

Table 39.4 Viability Determination of *Lis. monocytogenes* Strains in Inoculated Frankfurters (Low-Level Inoculation)

Treatment	Enumeration (log/g) after Storage at 4°C			Isolation (+/–) after Storage at 4°C		
	1 d	7 d	1 4d	1 d	7 d	14 d
Control (2)	3.2	5.6	6.3	2/2	2/2	2/2
BP (3)	ND[b]	0.5	3.6	3/3	3/3	3/3
HP (6)	ND	0.4	0.7	1/6	5/6	6/6
HP + BP (6)	ND	ND	ND	0/6	0/6	0/6

[a] See footnote of Table 39.3 for explanations. Number in parentheses indicates number of sample tested. Enumeration was done by plating on tryptic soy agar and isolation was done on moxalactam agar. Each package was opened and mixed with a selective enrichment broth. A portion was used for enumeration and the remaining materials in the package were incubated at 37°C for 1 to 2 d. A loopful of material was then streaked on a moxalactam agar plate, which after incubation for 48 h was examined for black characteristic colonies for *Listeria* survivors.

cheese inoculated before pressurization with 10^6/g each of *Bac. cereus* and *Clo. sporogenes* PA 3679. The product has a superior acceptance quality. This study has now been extended to several other foods. This HPP technique seems to be very effective in the production of different types of food products.

V. CONCLUSION

To meet consumer demand, several thermal and nonthermal technologies are being studied for the processing of foods. Among these, PEF and HPP, especially the HPP technology, offer a great potential. Both technologies are nonthermal methods and suitable for the production of pasteurized food. HPP, however, in combination with high heat can be used to produce commercially sterile foods that have a better acceptance quality than that of foods produced by the comparable thermal process. HPP can also be used to produce novel foods and novel processing techniques. Other parameters can be used with many of these technologies to provide an added advantage. This concept is discussed in Chapter 40 as the hurdle concept.

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QUESTIONS

1. List two thermal and four nonthermal processes among the currently studied technologies for application in food processing and preservation. What advantages do they have over the conventional methods now being used?
2. Briefly describe the principles of antimicrobial actions of the four nonthermal processes, other than PEF and HPP, listed in this chapter.
3. Briefly discuss the mechanisms of antimicrobial action of PEF technology and how this is being used to improve preservation of foods.
4. Discuss why HPP treatment does not adversely affect the food quality.
5. Describe six other applications of HPP in food processing besides preservation and discuss the advantages of the method in these applications.
6. Discuss the mechanisms of antimicrobial action of HPP against vegetative cells of microorganisms.
7. Discuss the problems associated with the sublethal injury and resistance of foodborne pathogens in pressure-processed foods.
8. Discuss the mechanisms by which foodborne bacterial spores can be destroyed at low and high hydrostatic pressure.
9. Define pressure pasteurization and pressure sterilization of food and discuss their differences from pasteurization and commercial sterilization of food by heat treatment.
10. List eight foods from four food groups that have been studied for microbial control in HPP.

CHAPTER 40

Control by a Combination of Methods (Hurdle Concept)

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I. INTRODUCTION

Many of the factors used to kill microorganisms or control microbial growth in foods are also able to facilitate microbial growth and survival. These factors, which include temperature, A_w, pH, Eh, and some preservatives, have a range in which microbial growth varies from optimal to minimal. Beyond the range, depending on the factor, microorganisms either do not grow or die. When only one method is used to preserve food, the condition beyond the growth range of microorganisms is generally used. Examples of such methods are high temperature treatment to produce a sterile food (preferably in a container to prevent postheat contamination) or drying a food below an A_w of 0.6. Although microbiologically these products can have a very long shelf life and be safe, their acceptance and nutritional qualities are generally lower and they might not be preferred by many consumers. This is particularly true for

consumers interested in foods that are fresh, natural, healthy, and convenient and not harshly processed or harshly preserved. There is a general interest in producing foods that are low in salt, sugar, and fat, and that can be stored for a desirable period of time by refrigeration or chilling or even at room temperature and be eaten readily or by microwave heating. There is also an interest to produce such fresh-like foods for the U.S. Army.

II. MECHANISMS OF ANTIMICROBIAL ACTION

Many foods invariably contain different types of spoilage bacteria, and there is a good chance that some can have pathogens (e.g., *Staphylococcus aureus* cells, *Listeria monocytogenes*, pathogenic clostridial spores). For safety and stability, the growth of pathogens and spoilage microorganisms must be controlled during the storage life expected for these products. To achieve this goal, two or more antimicrobial agents are employed together at a level at which the individual agents may facilitate some growth of the concerned microorganism but not when used in combination.[1,2] As each of these agents or methods is used "gently" (at a lower level) as opposed to "harshly," they normally do not adversely affect the acceptance and nutritional qualities of foods but do retain their desirable safety and stability. The mechanisms[1,2] by which the combination of factors, or hurdle concept, works can be explained with the following example (Figure 40.1A).

Two target microorganisms *a* and *b* can grow when preservation methods *X*, *Y*, or *Z* are used as individual hurdles. However, if *X* and *Y* are combined, the growth of *a* is arrested, and when *X*, *Y*, and *Z* are used in combination, both microorganisms fail to grow.

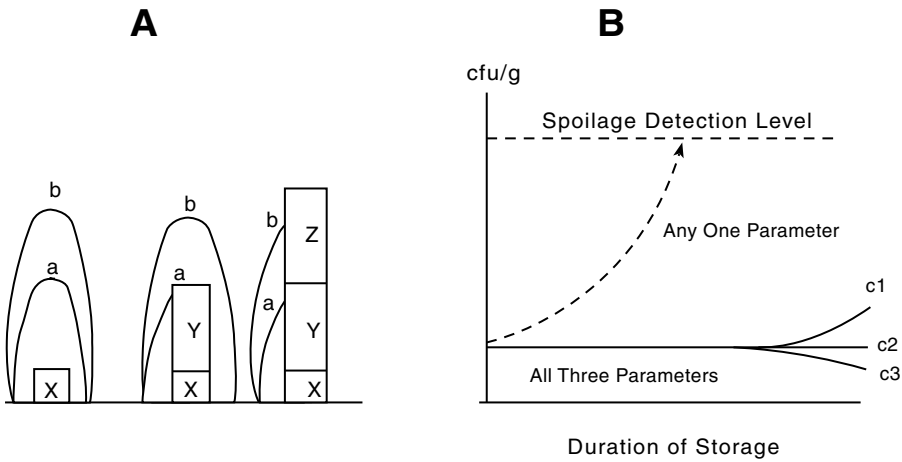


Figure 40.1 (A) Schematic presentation showing how the combination of several preservation techniques (X, Y, Z) is more effective in controlling different microbial groups (a, b). (B) An effective combination of several parameters can reduce the growth rate, prevent growth, or even kill microorganisms, which might not be achieved by using any one parameter.

The concept can also be explained with another example (Figure 40.1B). Suppose a food has several types of psychrotrophic spoilage bacteria that can grow rapidly at 30ºC or at pH 6.0 or at an A_w of 0.99 and spoil the food rather quickly. If any one of the factors is reduced (e.g., if the temperature is reduced to 5ºC, keeping the other two unaltered), the growth rate will be slightly reduced, but the product can still be spoiled before the desired shelf life time (say 60 d). However, if all three parameters are reduced slightly, which will allow the target microorganisms to grow if used individually, such as 5ºC, pH 5.0, and A_w 0.93, their growth will either stop (C_2) or occur at a very slow rate (C_1), or they might even show some death during storage (C_3). With this combination, the product may remain stable during its expected shelf life. Each of the three parameters used in this example can be ineffective individually, but when used together, their effect becomes additive, which the target organisms fail to accommodate for during growth. If more such parameters are added, the growth of microorganisms in foods can be reduced drastically for a considerable period of time.

The different parameters or factors that are used in combination include intrinsic factors (e.g., A_w, pH, Eh, and natural inhibitors), processing factors (e.g., heating, drying, fermentation, and preservatives), and extrinsic factors (e.g., temperature and aerobic or anaerobic environment). Competitive flora (e.g., lactic acid bacteria) and nonthermal processing methods (Chapter 39) can be added to them. Food preservation by combining several of these factors has been used for a long time. An example is the preservation of jams and jellies, for which high heat, low pH (of fruits), low A_w (sugar in fruits and added), and anaerobic packaging are used to reduce microbial numbers as well as the growth of survivors. However, the greatest challenge will be the long-term preservation of meat, dairy, and fish products at refrigerated temperature or even at ambient temperature, where refrigeration is not always available, such as during a military operation, a work relief during a natural disaster, or in the rural areas of many developing countries. To achieve this goal, it will be necessary to study the effectiveness of combining different factors to control target microorganisms in a food. In conducting such studies, one should also recognize that the factors could have additive, synergistic, or even adverse (neutralizing) effects when used in combination.

III. CURRENT STATUS

These aspects have been studied in a rather limited way. Results of some of these studies are briefly described here.[3,4]

A. Low-Heat Processing

Low heat (≤100ºC) does not kill many pathogenic and spoilage bacterial spores. They can be heat activated, which leads to germination and outgrowth. However, if the pH of the food is reduced to 4.5 or NO_2 and NaCl are added, the heat-shock spores will not germinate.

B. Low Storage Temperature

Clostridium botulinum grows at 35ºC at an A_w of 0.95. However, if the storage temperature is reduced to 20ºC, it does not grow unless the A_w is increased to 0.97. Similarly, *Lis. monocytogenes* grows at 25ºC in a broth containing 6.5% NaCl in 3 d, but fails to grow under similar conditions at 14ºC.

C. Low pH

Clo. botulinum grows at pH 7.0, 37ºC, and an A_w of 0.94; however, when the pH is dropped to 5.3, no growth is observed, even at an A_w of 0.99. A *Salmonella* strain grows at pH 5.8 at an A_w of 0.97, but when the pH is reduced to 5.0, an A_w of 0.99 is required for growth. *Clo. botulinum* produces a toxin during incubation at 16ºC in 28 d at pH 5.5, but at pH 5.2 under the same conditions, no toxin is produced.

In using acid to reduce pH, it is important to recognize that organic acids, such as acetic, propionic, and lactic acids, are more effective than HCl and phosphoric acid. Also, acetic or propionic acids are more effective than lactic acid. Some acids, such as citric and phosphoric acids, act by both lowering pH and chelating divalent cations that are important for microbial functions.

D. Low A_w

Some of the examples used show how a reduced A_w interacts with other parameters in adversely affecting microbial growth. *Sta. aureus* can grow at an A_w of 0.86 and high pH. However, it fails to grow even at an A_w of 0.93 (in the presence of NaCl) at pH 4.6, a pH which favors growth of this bacterium at higher A_w. Similarly, *Sta. aureus* grows at 12ºC, pH 7.0, and an A_w of 0.93. However, if the A_w is reduced to 0.90, it will not grow under the same conditions.

It is also important to recognize that at a low A_w, some microorganisms may develop resistance to the killing effect of heat.

E. Modified Atmosphere

In vacuum-packaged (N_2 or CO_2) foods, growth of aerobes is prevented and that of facultative anaerobes can be reduced. But there is a concern that vacuum packaging can selectively facilitate growth of anaerobic spoilage and pathogenic bacteria. Thus, suitable combinations of other factors, such as low pH, A_w, or preservatives, must be used with modified atmosphere to control their growth.

F. Preservatives

Some preservatives such as NaCl and BHA act synergistically to increase the antimicrobial action of sorbates. Organic acids are also effective at low pH because of higher concentrations of undissociated molecules. Some preservatives may not be effective at higher pH and some may lose potency during storage. Bacteriocins may be destroyed by proteolytic enzymes present in the food (raw food). The bactericidal effect of bacteriocins can be enhanced when used with acids, SDS, and EDTA.

G. Hydrostatic Pressure Processing (HPP)

The antibacterial effect of ultrahigh hydrostatic pressure can be increased by incorporating mild heat, slightly low pH, bacteriocins, lysozyme, and chitosan. Spores are difficult to destroy unless very high pressure is used (≅700MPa). However, after low-pressure treatment (≥100MPa), the spores may be induced to germinate, which can then be killed by higher pressure, mild heat, bacteriocins, or other antibacterial agents (see Chapter 39).

IV. CONCLUSION

Many of the control methods listed, such as low heat, low pH, low A_w, low temperature, and hydrostatic pressure, induce sublethal stress among the surviving cells and spores. Cells and spores injured by one parameter become sensitive to other parameters and are killed in their presence. Sublethal injury to microbial cells and spores and increased susceptibility of injured cells and spores to one or more preservation methods can play important roles in controlling microorganisms by a combination of factors.[5]

It will be important to study the beneficial effect of combining different parameters at different levels to determine their antimicrobial efficiency against target microorganisms in food systems. The hurdle concept is based on the scientific concept that when two or more methods are used together the combinations are more effective, even at a much lower treatment level. However, because of the large number of possibilities, actual product challenge studies may not be possible for all. Instead, at the initial stage, computer simulation by using multifactorial parameters can be designed that take into consideration the characteristics of a food. Then limited number of selected parameters can be studied by product inoculation. These methods are now being developed (see Appendix B).

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QUESTIONS

1. Describe the principle of using a combination of factors, or hurdle concept, in the preservation of "fresh-like" foods.
2. In combining two or more factors to preserve foods, what criteria need to be considered?
3. Give three examples to show how the combination of factors has been found to control growth of foodborne pathogens.
4. Spores of *Clostridium laramie*, associated with spoilage of roast beef at refrigerated temperature, were not destroyed by a UHP treatment of 80,000 psi for 20 min. In contrast, when the spores were subjected to a UHP treatment at 20,000 psi for 10 min and then treated with a bacteriocin containing biopreservatives and incubated at optimum temperature, no growth was observed. However, the pressurized samples without bacteriocin had growth. Explain the possible causes for the difference.
5. How can microbial sublethal injury be advantageously used to control microbial growth in food preserved by the hurdle concept?
6. Take a trip to your nearest supermarket and select six refrigerated foods that have been preserved (to control microbes) by using the hurdle concept.

SECTION VII

Appendices

This section includes brief discussions of topics that are considered either relatively new in food microbiology or taught in special and advanced food microbiology courses or in laboratory courses. Thus, these topics can be used as additional reading materials:

Appendix A: Microbial Attachment to Food and Equipment Surfaces
Appendix B: Predictive Modeling of Microbial Growth in Food
Appendix C: Regulatory Agencies Monitoring Microbiological Safety of Foods in the U.S.
Appendix D: Hazard Analysis Critical Control Points
Appendix E: Detection of Microorganisms in Food and Food Environment

APPENDIX A

Microbial Attachment to Food and Equipment Surfaces

I. IMPORTANCE

The normal tendency of a microbial cell when it comes in contact with a solid surface is to attach itself to the surface in an effort to compete efficiently with other microbial cells for space and nutrient supply and to resist any unfavorable environmental conditions. Under suitable conditions, almost all microbial cells can attach to solid surfaces, which is achieved through their ability to produce extracellular polysaccharides. As the cells multiply, they form microcolonies, giving rise to a biofilm on the surface containing microbial cells, extracellular polysaccharide glycocalyx, and entrapped debris. In some situations, instead of forming a biofilm, the cells may attach to contact surfaces and other cells by thin, thread-like exopolysaccharide materials, also called fimbriae.

Attachment of microorganisms on solid surfaces has several implications on the overall microbiological quality of food. Microbial attachment to and biofilm formation on solid surfaces provide some protection of the cells against physical removal of the cells by washing and cleaning. These cells seem to have greater resistance to sanitizers and heat. Thus, spoilage and pathogenic microorganisms attached to food surfaces, such as carcasses, fish, meat, and cut fruits and vegetables, cannot be easily removed by washing, and later they can multiply and reduce the safety and stability of the foods. Similarly, microbial cells attached to equipment surfaces, especially those that come in contact with the food, may not be easily killed by chemical sanitizers or heat designed to be effective against unattached microbial cells (like in a suspension), and thus they can contaminate food. This problem increases if cleaning and sanitation are delayed following equipment use. Finally, microbial attachment and biofilm formation in the food-processing environment, such as floors, walls, and drains, enable the cells to establish in the environment; and they become difficult to control effectively by the methods developed and designed against the unattached microorganisms from

a culture broth. These places, in turn, can be a constant source of undesirable microorganisms to foods handled in the environment.

The concept and importance of microbial attachment and biofilm formation in solid food, equipment, and food environments are now being recognized.[1–3] Limited studies have shown that under suitable conditions, many of the microorganisms important in food can form a biofilm. Several species and strains of *Pseudomonas* were found to attach to stainless steel surfaces, some within 30 min at 25°C to 2 h at 4°C. *Listeria monocytogenes* was found to attach to stainless steel, glass, and rubber surfaces within 20 min of contact. Attachment of several pathogenic and spoilage bacteria has also been demonstrated on meat and carcasses of poultry, beef, pork, and lamb. The microorganisms found to attach to meat surfaces include *Lis. monocytogenes, Micrococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Brochothrix thermosphacta, Salmonella* spp., *Escherichia coli, Serratia* spp., and *Pseudomonas* spp.. It is apparent from the limited data that microbial attachment to solid food and food contact surfaces is quite wide and needs to be considered in controlling the microbiological quality of food.

II. MECHANISMS OF ATTACHMENT

Several possible mechanisms by which microbial cells attach and form a biofilm on solid surfaces have been suggested.[1–3] One suggestion is that the attachment occurs in two stages. In the first stage, which is reversible, a cell is held to the surface by weak forces (electrostatic and Van der Waals forces). In the second stage, a cell produces complex polysaccharide molecules to attach its outer surface to the surface of a food or equipment, and the process is irreversible. A three-step process that includes adsorption, consolidation, and colonization has been suggested by others. In the reversible adsorption stage, which can occur in 20 min, the cells attach loosely to the surface. During the consolidation stage, the microorganisms produce threadlike exopolysaccharides fimbriae and firmly attach the cells to the surface. At this stage, the cells cannot be removed by rinsing. In the colonization stage, which is also irreversible, the complex polysaccharides may bind to metal ions on equipment surfaces and the cells may metabolize products that can damage the surfaces[1–3] (see cover page).

III. INFLUENCING FACTORS

The level of attachment of microorganisms to food-processing equipment surfaces is found to be directly related to contact time. As the contact time is prolonged, more cells attach to the surface, the size of the microcolony increases, and attachment between cells increases. Fimbriae formation by the cells occurs faster at optimum temperature and pH of growth. Limited studies also showed that when microorganisms such as *Pseudomonas fragi* and *Lis. monocytogenes* are grown together, they form a more complex biofilm than when either is grown separately.[1–3]

The factors associated with the attachment of spoilage and pathogenic bacteria to meat and carcass surfaces can be divided into three groups: associated with bacteria, associated with meat, and extrinsic factors.[1–3] Many Gram-positive bacteria (*Clostridium, Micrococcus, Staphylococcus, Lactobacillus* spp., and *Bro. thermosphacta*) and Gram-negative bacteria (*Esc. coli, Proteus, Pseudomonas, Serratia, Salmonella, Enterobacter, Shewanella,* and *Acinetobacter* spp.) can attach to skin and meat surfaces of chicken, pork, beef, and lamb. Some studies showed that several Gram-negatives, such as *Proteus* and *Pseudomonas* spp., attach more rapidly and in higher numbers than some Gram-positive bacteria, such as *Lactobacillus, Staphylococcus*, and *Micrococcus* spp. However, there are differences in observations, and some researchers think that the more negative charge the cells of species or strains have, the faster and higher is their ability to attach to muscle surfaces. Similarly, results differ among researchers with respect to influences of types of food animals, birds, and microbial species to attachment. Bacterial attachment to lean tissues is generally higher than to adipose tissues; however, there is probably no difference in the attachment of both Gram-positive and Gram-negative bacterial species in the skin and muscle of different types of food animals and birds. Among the extrinsic factors, the number of attached cells on meat surfaces is directly related to contact time and cell concentrations used. At optimum growth temperature, a bacterial species shows a higher attachment rate. Electrical stimulation of the carcasses may favor attachment, but opinions differ on this.[1–3]

IV. CONTROL MEASURES

Microbial biofilm formation on equipment surfaces and attachment to solid food surfaces can resist their removal and killing by some of the methods used but designed to remove or kill unattached microbial cells from a culture broth. Some modifications in these methods may be necessary to overcome the problems, especially in food contact and processing equipment surfaces. Biofilms are difficult to remove if they are left to grow. They should be removed when the biofilms are young. In a food-processing operation, cleaning and sanitation has to be done every few hours. This needs to be determined for a specific food and the nature of the operation. To break down the glycocalyx minerals, a treatment with some suitable hydrolyzing enzymes may be initially administered. EDTA, along with treatment by quaternary ammonium compounds following enzyme treatment, can be helpful. In addition, processing equipment needs to be designed to prevent or control biofilm formation. To reduce attachment of microorganisms to solid food surfaces, suitable control measures need to be adopted. Use of suitable preservatives, low A_w, low pH, and low storage temperature can be used judiciously in combinations to reduce the rate of biofilm formation in food.

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APPENDIX B

Predictive Modeling of Microbial Growth in Food

I. IMPORTANCE

Every year, more than 12,000 new food products are introduced to U.S. consumers in addition to the existing more than 100,000 food items. Some of these products obviously have limited shelf life because of the possibility of microbial growth under the conditions in which they are stored and handled following production. The initial microbial population in many of these products may include both spoilage and pathogenic types. To ensure stability and safety, it is important that proper control measures are used to prevent growth of the microorganisms from the time of production to the time of consumption of these products.[1,2]

Among the thousands of new products marketed every year, some are definitely produced to satisfy consumers looking for foods that are healthy, natural, low-fat, low-salt, tasty, convenient, do not contain harsh preservatives, are not given harsh treatment, have long storage life, and are safe. To produce these new types of foods (such as the new-generation refrigerated foods) with desirable safety, stability, and acceptance quality, the influence of many parameters, such as different ingredients at different concentrations under different processing and storage conditions and at different A_w, pH and other intrinsic and extrinsic factors, on microbial growth and destruction in foods has to be determined.

It is almost impossible to conduct such microbiological studies for each product by the methods traditionally used to ensure safety and stability of food products. However, with the aid of computers, mathematical models can be developed to determine the influence of combinations of several parameters on microbial growth. Although they may not be accurate, they can be effective to obtain first-hand information very rapidly, helpful to eliminate many of the less important possibilities, and select a few that appear more promising. This information can then be used to conduct a modest traditional study that is feasible both experimentally and economically.

II. TRADITIONAL METHODS

Several methods are used to determine the influence of control parameters on growth or toxin production, or both, by targeting microorganisms in foods. Three of these methods are briefly described here.[3,4]

A. Challenge Studies

In this method, a food is first inoculated with the microorganisms that are expected to be the major causes of loss of stability or safety, or both, under retailing conditions. A realistic number of microbial cells or spores is used as inoculum. The product is then stored under conditions in which it is expected to be stored normally and then examined for the duration of storage for microbial numbers (growth) or toxin production.

To determine effective control measures against the target microorganisms, a large number of variables need to be studied. These include ingredients used, intrinsic and extrinsic factors, possible abuse, and others. As a result, the study can be quite complicated, time-consuming, and costly.

B. Storage Studies

A normal product is stored under its proper storage conditions and, at selected intervals, samples are examined for the level of spoilage and pathogenic microorganisms that are expected to be normally present in the product and grow under the storage conditions. The results can be used to predict the expected shelf life and safety under the suggested storage conditions. However, this study will not provide information if the product is temperature abused even for a short time. In addition, if some of the samples being tested do not, by chance, contain the spoilage and pathogenic microorganisms normally expected to be present, then the results are of no practical value.

C. Accelerated Shelf Life Studies

For products expected to have a relatively long shelf life, accelerated tests provide rapid information about their storage stability. The products are generally held at a higher temperature in order to increase the rate of microbial growth and accelerate the spoilage. However, foods normally contain mixed microflora that differ in optimum growth rate at different temperature, and spoilage of a food is generally caused by a few species, among those present, that grow optimally at a given condition. To make the results valid, in designing this study, the temperature should be increased only to a level that does not affect the growth of the microorganisms of interest.

III. PREDICTIVE MICROBIOLOGY

Several mathematical models have been developed to predict growth of pathogenic and spoilage microorganisms in foods from the data generated by studying microbial

growth rate at different pH, A_w, temperature, and preservative concentrations in laboratory media. The availability of suitable computers has helped in the rapid analysis of multifactorial data. Two kinetic-based models are discussed here. They indicate the effect of culture parameters on the growth rate of a microorganism, especially at the lag and exponential growth phases.[1–4]

A. Square Root Model

This is based on the linear relationship between the square root of the growth rate and temperature, and is expressed as: $\sqrt{r} = b\,(T - T_{\min})$, where r is rate of growth, b the slope of the regression line, T the temperature of food, and $T_{\min}$ the theoretical minimum temperature of growth. The model has been further expanded to include the effects of temperature, pH, and A_w on growth rate:

$$\sqrt{r} = b\,\sqrt{[(A_w - A_{w\ \min})(\mathrm{pH} - \mathrm{pH}_{\min})]\,(T - T_{\min})}$$

where A_w and pH are water activity and pH of a food, respectively, and $A_{w\,\min}$, $\mathrm{pH}_{\min}$, and $T_{\min}$ are minimum or lower limits of A_w, pH, and temperature, respectively, for growth.

In general, this model is quite effective when one or two parameters are used. However, its effectiveness is reduced when a variety of parameters, such as pH, organic acid, modified atmosphere, A_w, preservatives, salts, and others are used in combination to control microbial growth.

B. Sigmoidal Model (Gompertz: USDA Model)

This model has been developed by the U.S. Department of Agriculture (USDA) to predict microbial growth in a food environment containing many controlling parameters. It has been tested in laboratory media to determine the growth rate of *Listeria monocytogenes, Staphylococcus aureus, Salmonella* spp., *Shigella flexneri, Escherichia coli* O157:H7, and *Aeromonas hydrophila* under variable temperatures, pH, A_w, NaCl and $NaNO_2$ concentrations, and aerobic and anaerobic atmosphere. The growth curves were statistically fitted using nonlinear regression analysis in conjunction with Gompertz functions. The results were then analyzed to develop this model. It gives the lag time, the maximum growth rate constant, and the maximum microbial load directly from nonlinear regression of the numbers vs. time data. For these four parameters, the asymmetric sigmoidal equation is:

$$N = A + C\,\exp(-\exp\,[-B(t - M)])$$

where N is $\log_{10}$ colony-forming units (CFUs) per milliliter at time t, A the initial $\log_{10}$CFUs/ml, C the $\log_{10}$CFU difference between time t and initially, exp is exponential, B the relative growth rate at M, and M the time at which growth rate is maximum. This model has been extensively used because of its simplicity and overall effectiveness.

IV. CONCLUSION

Predictive microbiology has generated tremendous interest because of its many advantages, most important of which is the quick availability of computer-generated information for both simple and complex studies. Interactions between different intrinsic and extrinsic factors and the importance of changing a parameter on growth and survival of microorganisms can be determined very quickly. However, current models have been designed by using laboratory media. Thus, their effectiveness in different food systems is not exactly known. Limited studies have shown that in a food system (e.g., ham salad), the growth responses of *Listeria* spp. and *Salmonella* spp. obtained by predictive modeling and by actual experiment were favorable, whereas for *Sta. aureus* the growth rate predicted by the model was lower than that obtained in actual experiments. Thus, at present, the predictive data needs to be adopted with caution. Model data can be used in initial studies by using large numbers of variables to eliminate a great majority of them. Experimental data can then be obtained from actual product studies by using limited numbers of variables. This will help reduce the time and cost and make the study more manageable and effective.

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APPENDIX C

Regulatory Agencies Monitoring Microbiological Safety of Foods in the U.S.

I. FOOD SAFETY REGULATIONS

Annual sales of food in the U.S are more than $400 billion. Before foods reach the consumers, they are processed and handled conservatively by more than 25,000 processors, 35,000 wholesalers, 250,000 retailers, 500,000 eating establishments, and in many millions of homes. If one adds to them the various types of pathogens that can cause foodborne diseases and their natural presence in food animals and birds, food-producing environments, and among food handlers, it becomes quite apparent that the chances of a foodborne pathogen being present in a food are high. Yet the numbers of reported foodborne disease outbreaks and the cases per year are relatively low. A major reason for this could be the stringent food safety laws in the U.S.[1,2]

Before 1906, there were no food laws in this country and marketing of unsafe food was quite common. In 1906, the federal government passed the Pure Food and Drug Act and the Meat Inspection Act and gave the U.S. Department of Agriculture (USDA) the power of supervision over the laws. According to these laws, selling of unwholesome and unsafe foods and meat in interstate commerce was illegal. The Meat Inspection Act also required inspection of the slaughter and meat-processing facilities. However, as the burden of proof that a substance had been added to a food at an unsafe level was with the USDA, the laws were not very effective. In 1938 the federal government passed the Food, Drug, and Cosmetic (FD&C) Act and gave additional power to the Food and Drug Administration (FDA) to administer the law. This law prevents the manufacture and shipment of unsafe and spoiled food and food ingredients in interstate commerce as well as for export and import. It permits sale of foods that are pure, wholesome, safe, and produced in a sanitary environment. According to this law, contamination of certain foods with specific pathogens, such as *Clostridium botulinum* and *Salmonella* spp., is considered to be unwholesome and selling such food is illegal. (At present several other potential foodborne pathogens, such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* in ready-to-eat foods, are given the same status.)

In 1959, the Federal Poultry Product Inspection Act was passed. This act requires the inspection of poultry-processing facilities that ship products in interstate commerce.

The Wholesome Meat Act (1965) and the Wholesome Poultry Act (1968) were passed, requiring inspection of the processing facilities, even those engaged in intrastate commerce. These facilities are to be inspected by state inspectors in the same manner as the processors engaged in interstate commerce and inspected by federal inspectors. At the federal level, the inspection of meat, poultry, and eggs, and milk remains with the Food Safety and Inspection Service (FSIS) branch in the USDA.[1,2]

II. THE AGENCIES

A. Federal Agencies

In the U.S., several federal agencies are delegated the responsibilities to monitor and regulate the origin, composition, quality, quantity, safety, labeling, packaging, and marketing of foods. Two agencies, the FDA and USDA, are directly involved with the microbiological safety of foods, and their responsibilities in this regard are discussed here.[2] Other agencies are involved in areas not directly related to microbiological safety of foods. Some of these are:

1. *Bureau of Alcohol, Tobacco and Firearms (ATF).* Responsible for enforcing the laws that cover production and labeling of all alcoholic beverages except wine.
2. *Department of Justice*. Conducts seizures of a product and criminal proceedings in case of a violation of food law.
3. *Environmental Protection Agency (EPA)*. Determines safety and tolerance levels of pesticide residues in foods and establishes water quality standard for drinking water (not bottled water).
4. *Federal Trade Commission (FTC)*. Regulates correct procedures of advertising of foods.
5. *National Marine Fisheries Service (NMFS)*. Responsible for seafood quality, habitat conservation, and aquaculture production (not microbiological quality).
6. *FDA*. At present, the FDA is a part of the Federal Public Health Service in the Department of Health and Human Services. It is delegated with the power to administer the federal FD&C Act. In that capacity, the FDA ensures the safety and wholesomeness of all foods, except fresh meat, poultry, and eggs sold in interstate commerce. It also oversees the wholesomeness of all exported and imported foods (except for fresh meat, poultry, and eggs). The FDA also inspects the safety and wholesomeness of foods served on planes, trains, and buses. It also conducts research to improve detection and prevention of contamination of food; develops regulatory testing procedures, specifications, and standards; and enforces regulations on processing and sanitation of food and processing facilities. The FDA sets up specific microbiological criteria that contain specifications (toleration levels) for aerobic plate counts (APCs), coliforms, *Esc. coli*, coagulase-positive *Staphlococcus aureus*, pathogens, and mycotoxins in various foods. FDA inspectors inspect processing plants once in two to three years, unless a processor is in

violation and a food has hazard potential. The FDA also helps processors develop their effective sanitation programs and gives contracts to state regulatory agencies to inspect plants. For foreign countries interested in exporting food to the U.S., the FDA sends inspectors to help improve their food quality according to U.S. regulations. In case of violations, the agency sends formal notice of violation to the offender and sets a date (usually 10 d) by which the problem needs to be corrected. In case of failure, depending on the severity of the offence, the FDA is authorized to take legal actions, through the Department of Justice, which include seizure of products, destruction of products, and criminal prosecution of the offenders. This includes products produced in the U.S. as well as imported products. In addition to microbiological quality and safety of foods, the FDA is also responsible for testing foods for composition, nutritional quality, food additives, food labeling, and pesticide residues (some with other agencies). The FDA also conducts market basket surveys to test a certain number of foods (imported and produced in the U.S.) for safety and other qualities.

7. *USDA*. Under the Wholesome Meat Act and the Wholesome Poultry Product Act, the FSIS of the USDA is responsible for inspecting slaughtering facilities of food animals and birds, and producing wholesome and safe meat, poultry, eggs, and egg products that are sold in interstate commerce or imported. The FSIS also sets up microbiological specifications, particularly pathogen levels, for some of these products and conducts research on food safety and quality. Based on eating quality, the USDA also grades vegetables, fruits, milk and dairy products, and meat and egg products either produced in the U.S. or imported.
8. *Centers for Disease Control and Prevention (CDC)*. The CDC is a branch of the Department of Health and Human Services. In the event of a foodborne disease outbreak, this agency investigates the causative agent of the outbreaks as well as the sequence of events that resulted in the outbreak.
9. *U.S. Army*. The wholesomeness and safety of foods (ration) consumed by the U.S. military personnel are the responsibility of the Department of Defense. The microbiology branch at Natick Army Research facility has established necessary microbiological specifications for APCs, coliforms, *Esc. coli*, pathogens, and other microbial groups for different types of foods procured from outside suppliers.

B. State and Local Government Agencies

Some of the branches in the State Department of Agriculture and Public Health are responsible for the safety of food sold in the state. They cooperate with the federal government agencies to ensure wholesomeness and safety of foods produced and served in the state. State inspectors inspect restaurants, retail food stores, dairies, grain meals, and processing facilities on a regular basis. Some states have authority over inspection of the quality of fish and shellfish taken from state waters. These agencies, if necessary, can embargo illegal food products sold in the state. Federal agencies provide guidelines, when necessary, to state agencies for the regulations.

C. International Agencies

The Food Standard Commission of the Joint Food and Agricultural Organization and World Health Organization (FAO/WHO), in cooperation with different nations,

helps develop international standards in the production, processing, and preservation of foods exported and imported. Codex Alimentarius (CA) is an international food regulatory agency formed by different nations. It helps develop uniform food standards for all countries to ease export and import of foods between countries.

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APPENDIX D

Hazard Analysis Critical Control Points

I. INTRODUCTION

The traditional method of examining microbiological safety, storage stability, and sanitary quality of a food is to test representative portions (or samples) of the final product for the presence of some pathogens (such as *Salmonella*) or the number or level of certain pathogens (e.g., *Staphylococcus aureus* and *Vibrio parahaemolyticus*), different microbial groups (e.g., aerobic plate counts, psychrotrophic counts, thermoduric counts, and yeasts and molds), and indicator bacteria (e.g., coliforms and enterococci) per gram or milliliter of product. The major disadvantage with these types of end-product testings is that they do not provide close to 100% assurance about the safety, stability, and sanitary quality of the products. This aspect is particularly important with respect to pathogenic bacteria, especially those that are highly potent. By analyzing samples from a batch of a product, according to sampling plan and testing methods recommended by regulatory agencies (see Appendix E), it is not possible to give a high degree of assurance (close to 100%) that the untested portion of the end product is free of pathogens. Under certain circumstances, such as foods consumed by the crew in a space mission or by military personnel engaged in an important mission, close to 100% assurance of safety of a food is required.

Hazard analysis critical control points (HACCP) was originally developed jointly by the Pillsbury Company, the National Aeronautics and Space Administration (NASA), and the U.S. Army Natick Laboratories to produce foods with high assurance of safety for use in the space program. It has two components: (1) to identify or analyze the hazards (HA) associated with the production and processing of a specific type of food (i.e., which pathogens can be expected to be present in a food), and (2) to identify critical control points (CCP) (i.e., the places during processing of a food where proper control measures need to be implemented in order to prevent any risk to consumers). It is regarded as a systemic approach to assure safety and as a better method than end-product testing.

Since the introduction of the concept in 1971, HACCP has undergone several changes according to specific needs. In 1980, the National Academy of Science suggested that food industries in the U.S. could use the HACCP principles to produce safer foods and regulatory agencies could help develop programs suitable for a particular processing operation. Currently, the Food Safety and Inspection Service (FSIS) of the USDA advocates the use of HACCP in meat and poultry inspection programs during both slaughtering and processing of carcasses for products. The Food and Drug Administration (FDA) is also developing HACCP programs that cover various products it regulates. Internationally, HACCP is used in Canada, Australia, New Zealand, and several European countries.

It seems that the HACCP concept can be used universally to develop food safety programs. This will help reduce problems associated with foodborne pathogens as well as global trade of foods.[1–3]

II. HACCP PRINCIPLE OF THE NACMCF

There have been some changes in the original HACCP system. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has developed a document for the HACCP system.[1–3] It is expected that by implementing this system in the production and processing of food, the safety of the products can be assured. This system is briefly presented here.

A. Seven Principles of HACCP

HAACP involves seven principles:

1. Conduct a hazard analysis to determine risks associated at all stages, from growing raw materials and ingredients to final product ready for consumption.
2. Identify critical control points to control these hazards.
3. Implement conditions to control hazards at each critical control point.
4. Implement effective procedures to monitor control for each point.
5. Implement corrective measures to be taken if a deviation occurs at a point.
6. Implement effective record-keeping systems for HACCP plan activities.
7. Implement procedures to verify that the plan is working effectively.

B. Brief Description of the Principles

1. Principle 1

The possible hazards that exist for a specific food or ingredient from pathogenic microorganisms and their toxins are determined. This is performed in two segments: (1) by ranking the specific food into six categories and (2) by assigning each a risk category on the basis of ranking for hazard categories.

Ranking for six hazard categories is based on the following assessments for a food: (1) does it contain microbiologically sensitive ingredients; (2) does the processing method contain a step that is effective in destroying the pathogen; (3) does the processing method have a step for post-processing contamination of the product

with pathogens or their toxins; (4) is there a possibility of abusing the product during subsequent handling (transportation, display at retail stores, handling and preparation by the consumers) that can render the product harmful for consumption; and (5) is the product, after packaging, given an effective heat treatment before consumption?

The hazard characteristics of a food are based on the following:

Hazard A. A nonsterile food intended for consumption by high-risk consumers (infants, elderly, sick, and immunocompromised individuals).
Hazard B. The product contains "sensitive ingredients" in terms of microbial hazard.
Hazard C. The process does not contain a step to destroy harmful microorganisms.
Hazard D. The product is subject to recontamination after processing and before packaging.
Hazard E. The possibility exists for abuse before consumption that can render the product harmful if consumed.
Hazard F. The product is not given a terminal heat treatment following packaging and before consumption.

A food is ranked according to Hazards A through F by using a plus (+) for each potential hazard. The risk category is determined from the number of pluses.

Category VI. A special category that applies to nonsterile food to be consumed by high-risk consumers.
Category V. A food that has five hazard characteristics: B, C, D, E and F.
Category IV. A food that has a total of four hazard characteristics in B through F.
Category III. A food that has a total of three hazard characteristics in B through F.
Category II. A food that has a total of two hazard characteristics in B through F.
Category I. A food that has a total of one hazard characteristic in B through F.
Category *O*. No hazard.

2. Principle 2

To control microbiological hazards in a food production system, it is necessary to determine a CCP. A CCP is any point or procedure in a specific food system where effective control must be implemented in order to prevent hazards. All hazards in a food production sequence, from growing and harvesting to final consumption, must be controlled at some point(s) to ensure safety. The CCP includes heat treatment, chilling, sanitation, formulation control (pH, A_w, preservatives), prevention of recontamination and cross-contamination, employee hygiene, and environmental hygiene. Application of one or more CCPs will destroy hazardous microorganisms or prevent their growth and toxin formation in the product.

3. Principle 3

To control microbiological hazard at each identified CCP, it is necessary to set up critical limits (such as temperature, time, A_w, pH, preservatives, and, in some cases, aroma, texture, and appearance). One or more critical limit may be necessary at each CCP, and all of them should be present to ensure that the hazards are under control.

4. Principle 4

The CCP used for a specific food production must be monitored to determine whether the system is effective in controlling the hazards. The monitoring can be continuous, such as in case of heat treatment (temperature and time) of a product. If continuous monitoring cannot be adopted for a processing condition, it can be done at reliable intervals. The interval for a food production has to be developed on a statistical basis to ensure that the potential hazards are under control. Most online monitoring is done by rapid chemical and physical methods. Generally, microbiological methods are not effective because of the long time required for testing. However, some current methods, such as the bioluminescence method, could be effective under some conditions. All results of monitoring must be documented and signed by persons doing the monitoring as well as by an official of the company.

5. Principle 5

In case a deviation from the present HACCP plan is identified from the monitoring system in a food production operation, effective corrective actions must be taken to assure product safety. The corrective actions must demonstrate that it is effective in controlling the potential hazards resulting from the deviation of CCP. The deviations as well as the specific corrective measures for each must be documented in the HACCP plan and agreed on by the regulatory agency before applying the plan. Also, the product produced by the new plan should be placed on hold until its safety has been ensured by proper testing.

6. Principle 6

Records of the HACCP plan developed for a specific food production should be kept on file in the plant. In addition, documents of CCP monitoring and any deviation and corrective procedures taken should be kept at the establishment. If necessary, the records must be made available to the regulatory agencies.

7. Principle 7

Verification systems should be established to ensure that the HACCP system developed for a specific food production system is working effectively to ensure safety to the consumers. Both the food producer and the regulatory agency have to be involved in the verification of the effectiveness of the HACCP in place. Verification methods include testing samples for physical, chemical, sensory, and microbiological criteria as established in the HACCP plan. The NACMCF adopted guidelines for HACCP and its application. In it, the wording of the seven principles was condensed and the meanings kept unchanged. It also provided the specific definition of terms such as hazard, verification, and validation; included sections on prerequisites, training, implementation and maintenance; and gave a detailed explanation of the application of the HACCP program.[4]

III. CONCLUSION

In a biological hazard category, the main objective of the HACCP system is to provide a high degree (close to 100%) of assurance that a food ready to be consumed will be free of pathogenic microorganisms or their toxins. However, the same principle can be applied to design control of spoilage microorganisms in foods, especially for those expected to have extended shelf life. Currently, there is an increase in the production of vacuum- and modified-air-packaged unprocessed and low-heat-processed food products, many with low-fat content and high pH, some with an expected shelf life of more than 50 d at refrigerated temperature. There is evidence that many of these products show unusual types of microbial spoilage and some are by species that were not known before (see Chapter 20). Although studies are limited, there is evidence that the incidence of such spoilage is on the rise. It would be commercially advantageous for the food industry to adopt HACCP not only for pathogens but to also control spoilage microorganisms. It is also logical to think about controlling microbiological problems as a whole, which would include both pathogens and spoilage microorganisms (i.e., total microbial quality, TQM). One cannot devise methods to control only pathogens or only spoilage microorganisms. The International Organization of Standardization (ISO) 9000 series standards can be used to supplement HACCP systems and develop effective process control and process assurance systems.[5] Finally, the HACCP system can be combined with the hurdle concept of food preservation for the effective control of pathogenic and spoilage microorganisms for foods that otherwise could not be produced commercially (see Chapter 40).[6] Although food safety is of primary importance in countries (especially developing countries) with abundant food supplies, food spoilage is equally important in many countries where large quantities of food are lost because of spoilage. In those countries, the HACCP concept to reduce food spoilage will be particularly important to increase food availability.

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APPENDIX E

Detection of Microorganisms in Food and Food Environment

I. IMPORTANCE

Microbial population in foods, food ingredients, and the food contact environment normally constitute many different species coming from different sources. The total microbial population in a food varies greatly, depending on the level of sanitation used at all phases, the degree of abuse that leads to microbial growth, and the processing and preservation methods used to kill and prevent growth of microorganisms. Similarly, contamination of a food by specific types or species of microorganisms depends on the presence or source of these microorganisms and their entrance into the food, mostly due to poor sanitation, during handling and processing. Microbiological examination of foods and food ingredients helps assess their safety to consumers, their stability or shelf life under normal storage conditions, and the level of sanitation used during handling. In addition, the microbiological load and type can be important in determining whether a food and food ingredient meet acceptable standards, specifications, and guidelines. Microbiological evaluation of raw materials also provides important information about the heat-processing parameters that would be necessary to meet the microbiological standard, guideline, or specification of a product. Finally, microbiological evaluation of a food, food ingredient, and environment helps determine possible sources of a specific microbial type in a food and, in the case of a heated food, the source and nature of postheat treatment contamination.

II. METHODS USED

The methods used for the microbiological evaluation or detection of foods, food ingredients, and environment are broadly grouped as quantitative and qualitative methods. Quantitative methods are designed to enumerate or estimate directly or indirectly the microbial load in a test material. None of the quantitative methods

used now enumerate or estimate "total microbes," "total bacteria" or "total viable population" (terms used by many) present in a food, although it can be obtained for a pure culture of a microbial strain growing in a sterile liquid or a solid medium. Rather, each method enumerates or estimates a specific group among the total microbial population normally present in a food and that grows or multiplies preferentially under the conditions or methods of testing. These include composition of an enumeration medium, temperature and time of incubation and oxygen availability during incubation, pH, and treatments given to a sample before enumeration and estimation. Examples of some of the quantitative methods used are aerobic plate counts (APCs, or standard plate counts, SPCs, for dairy products), anaerobic counts, psychrotrophic counts, thermoduric counts, coliform counts, *Staphylococcus aureus* counts, and yeasts and molds counts.

In contrast, qualitative methods are designed to determine whether a representative amount (a sample) of a food or a certain number of samples in a batch of a food contain a specific microbial species among the total microbial population. These methods are used to detect the possible presence of certain foodborne pathogens, especially those capable of inflicting high fatality rates among consumers. *Salmonella, Clostridium botulinum, Escherichia coli* O157:H7, and probably *Listeria monocytogenes* in ready-to-eat food, are some that fall into this group.

III. STANDARD AND RECOMMENDED METHODS

Many methods and variations of different methods that can be used for quantitative and qualitative detection of microorganisms in foods are reported in the literature. However, it is desirable to use methods that have been approved by regulatory agencies. They can be either standard or recommended methods. In the U.S., for the microbiological examinations of milk and milk products, one needs to use the methods that have been designated as standard by regulatory agencies. For other foods, however, specific microbiological methods have been recommended or approved by regulatory agencies. Although it is advisable to use such a method, there is no mandatory requirement. Some of the books in the U.S. approved by regulatory agencies are *Standard Methods for the Examination of Dairy Products*; *Standard Methods for the Examination of Water and Waste Water*; *Standard Methods for the Examination of Seawater and Shellfish*; *Compendium of Methods for the Microbiological Examination of Food* (all four are published by the American Public Health Association, Washington, D.C.), and *Bacteriological Analytical Manual of Food and Drug Administration* (prepared by the FDA and published by the Association of Official Analytical Chemists, Arlington, VA). The last two publications include recommended methods. All these books are revised when necessary to incorporate the new methods and to update old methods by different groups of expertise. Many other countries have similar publications for the standard and recommended methods. Similar books are published by international organizations, such as specific branches of the World Health Organization and the Food and Agricultural Organization of the United Nations.

A food microbiology course invariably contains a laboratory component. The methods described in one or more of these books for the microbiological examination of food, food ingredients, and environment can be used in the laboratory. This will help the students or interested individuals become familiar with the standard and recommended methods approved by regulatory agencies in the U.S. Some of the methods are briefly discussed here. Details of these methods are available in the books listed previously and references at the end of the appendix.

IV. SAMPLING FOR MICROBIAL ANALYSIS

A. Sample and Sampling Plan

The microbiological quality of a batch or a lot of a food, which could be liquid, solid, or semisolid, is determined by testing a small portion of the total amount. This portion tested is called a sample and it must be the true representative of the total mass. It is very important to develop and implement an effective sampling plan; otherwise, even the most sensitive testing method will not provide information about the microbiological quality of a food. This is particularly important for the highly virulent pathogens, such as *Salmonella* or *Esc. coli* O157:H7 or a potent toxin such as botulin. In addition, different laboratories testing the same product but from samples collected differently could give different results, thus making it difficult for regulatory agencies to decide whether to accept or reject a lot to ensure safety to the consumers.[1]

Several statistical sampling plans have been described in standard or recommended methodology books. Four of these are briefly discussed here.[1–3]

1. *Single Attribute Plan.* In this plan, a lot is sampled once and tested for the bacterium or bacteria of interest. The results are used to determine whether a lot should be rejected or accepted (on the basis of the presence or absence of *Esc. coli* O157:H7). The sample size or number of sample (n) to be tested is dependent on the batch or lot size (N). Defective units (C) of n are dependent on the microbial type.
2. *Multiple Attributes Plan.* Initially, based on the lot size (N), the number of samples (n_1) are tested. If the defective units (C_1) are above the acceptance level, a second sampling is performed (n_2), tested, and analyzed for defective units (C_2) to decide whether to accept or reject the lot.
3. *Three-Class Attribute Plan.* Microbial levels are divided into three groups: (1) $\leq m$ is acceptable, (2) $>m$ but $\leq M$ is marginal, and $>M$ is unacceptable. Number of units (C_1) allowed between $>m$ and $\leq M$ among samples tested (n) is defined for a microbial type.
4. *Plan for Low Level of Contamination.* This is important for some highly potent pathogens. Units, depending on the lot size, are tested to determine presence or absence of pathogens to make the decision.

B. Sampling Procedure

The individual collecting the samples should know why the samples are being collected, i.e., what types of testing will be conducted, such as for APC, coliforms, or a specific pathogen.[1] The person should also have information about the product if it has been implicated in a foodborne outbreak and the nature of the product (liquid, solid, or semisolid; frozen; bulk or single units). The sample should be collected by using proper sanitary measures to prevent any contamination. Following collection and until tested, the samples should not be handled to avoid growth or death of microorganisms. If the product is frozen, samples should be kept frozen until analyzed. Otherwise, they can be stored at 0 to 4°C. Each sample should be labeled to identify date, time, nature of sample, and types of analysis to be conducted, and the persons who collected the sample. The samples should be transported to the laboratory under conditions that avoid microbial contamination, growth, or death.

The laboratory that will test the samples should examine the conditions, such as temperature, appearance, and the sampling information, and note the time of receiving the samples. Once received in good condition, a sample should be tested as soon as possible. Standard or recommended methods should be used to prepare the sample and the procedures of testing for a specific microorganism or microbial group. The unused portion of the sample should be stored under proper conditions until the results are available. In some specific situations, a sample might need to be preserved for a considerable period of time (e.g., in an outbreak involving a lawsuit). The results should be recorded immediately and properly in permanent form.

V. QUANTITATIVE METHODS FOR MICROBIAL ENUMERATION IN FOODS

A. Direct Enumeration

1. Microscopic Counts

Either stained cells under a bright field or live cells under a phase-contrast microscope can be counted and, using an appropriate microscopic factor, these counts can be expressed as microscopic counts per milliliter or gram food sample. However, viable and dead cells cannot be differentiated by this method. In addition, a sample must have large numbers ($\cong 10^6$/ml, /g) of microorganisms for effective use of this method.[1–3] Foods that have particles cannot be effectively tested for enumeration microscopically.

2. Colony-Forming Units (CFUs) in Nonselective Agar Media

Aliquots from selected dilutions of a serially diluted sample (generally based on the number expected in a food; see Chapter 4) are either pour plated or surface plated by using nonselective media such as plate count agar (PCA), tryptic soy agar, or nutrient agar. Different media can give different results. However, PCA is recom-

mended for CFU determination of several groups. The temperature and time of plate incubation required for the colonies to develop differ with the microbial groups being enumerated. For SPCs, they are 32°C for 48 h; APCs, 35°C for 48 h; and for psychrotrophic counts, 7°C for 10 d or 10°C for 7 d. The same procedure with specific modifications can be used to determine thermophilic, thermoduric, and anaerobic groups present in a food sample. The specific groups to be tested depend on their relative importance in a food. For a vacuum-packaged refrigerated food, the most important groups will be psychrotrophic, anaerobic, and facultative anaerobic groups (see Chapter 20).[1–3]

3. CFUs in Nonselective Differential Media

A nonselective medium is supplemented with an agent capable of differentiating the colonies produced by specific groups of microorganisms that differ in metabolic or physiological characteristics from one another in the population. pH indicators or O–R indicators are often used in the medium. Thus, the colonies of cells capable of metabolizing lactose to lactic acid are differentiated from those that do not ferment lactose by growing them in an agar medium supplemented with lactose as a C source and a pH indicator such as bromocresol purple. The lactose-fermentating colonies will be yellow and the others that can not ferment lactose yet grow will be white against a purple background. Differential methods are also used in specific media enumerating proteolytic, lipolytic, and pectinolytic microbial groups in a food.

4. CFUs in Selective Agar Media

A medium can be supplemented with one or more selective or inhibitory agents and used by pour or surface plating of serially diluted samples. In the presence of such an agent, only the microorganisms resistant to it can grow. Incubation conditions to stimulate colony formation differ with the organisms being studied. Enumeration of aciduric bacteria in a medium at pH 5.0, yeasts and molds in a medium at pH 3.5, and *Clo. perfringens* in the presence of cycloserine are examples of selective enumeration of specific groups present in a food. Halophilic and osmophilic microorganisms can also be enumerated similarly by specific selective procedures.[1–3]

5. CFUs in Selective-Differential Agar Media

In this method, a medium is supplemented with one or more selective agents to allow selective growth of specific resistant microbial groups while inhibiting growth of other sensitive associative microorganisms. In addition to selective agents, a medium is also supplemented with agents that enable each type among the selective microbial groups to produce colonies that differ in characteristics from one another. Violet red bile agar for coliforms, KF-azide agar for *Enterococcus* spp., V-J agar or Baird-Parker agar for *Sta. aureus*, and media recommended for the enumeration of some pathogens (e.g., *Yersinia enterocolitica, Campylobacter jejuni, Salmonella* spp., *Lis. monocytogenes, Clo. perfringens, Aeromonas hydrophila*) are selective as well as differential agar media. Because of the presence of one or more selective

compounds, they allow selective growth and colony formation of several closely related species. The differential agents then help differentiate these species or groups from one another by their specific colony characteristics.[1–3]

B. Indirect Estimation

1. Dilution to Extinction in Nonselective Broths

The method consists of serially diluting a sample and transfer of an aliquot (usually 1.0 ml or 0.1 ml) to a final 5 or 10 ml of a nonselective broth, such as tryptic soy broth. This is followed by incubating the tubes at a specific temperature for a specific period of time, which depends on the specific microbial group being investigated. The tubes are then examined for the presence and absence of growth (from the turbidity of the broth). With the highest sample dilution that gave growth and assuming that this tube had one to nine viable cells of the group of interest, microbial numbers per milliliter or gram sample are estimated. The estimated numbers, however, can vary widely from the actual numbers. This method is not used much for the microbiological estimation in food.[1–3]

2. Most Probable Number (MPN) in Selective Broth

In this method, aliquots from a serially diluted sample are inoculated in a broth (in tubes) having one or more selective agents that facilitate growth of selected microbial groups present in a food. Generally, three or five broth tubes in each dilution and a minimum of three consecutive dilutions are used. After incubating at recommended temperature and time, the broth tubes in each dilution are scored for the presence and absence of growth. From the number of tubes showing growth in each of the three successive dilutions, the number of viable cells of the specific microbial group can be estimated from the available statistically calculated tables. This method also gives wide variation. MPN methods are used quite often to estimate coliforms and fecal coliforms in foods and water by using brilliant green lactose bile broth and E-C broth, respectively.[1–3]

3. Dye Reduction Test

The method is based on the principle that some dyes such as methylene blue and resazurin are colored in oxidized states but colorless under reduced conditions. This change can occur because of microbial metabolism and growth. It is assumed that the rate of reduction during incubation of a specific concentration of methylene blue added to a food is directly proportional to the initial microbial load in the food. However, because microbial groups in a mixed culture in a food differ in the rate of metabolism and growth and ability to reduce the environment, this method is not considered very accurate and effective with different foods. This method is generally used to determine the microbiological quality of raw milk.[1–3]

C. Enumeration of Injured Microbial Groups by Selective Media

Sublethally injured coliforms and pathogenic bacterial cells, when enumerated by selective agar media, may die because of their developed sensitivity to selective agents in the media and thus cannot be detected (see Chapter 9). They are first allowed to repair the injury in nonselective media (broth or agar) for a short period, which enables the cells to regain resistance to the selective agents. Following repair, they can be exposed to selective media. For enumerating coliforms in food that may contain injured coliforms, the diluted sample can first be pour plated in nonselective plate count agar and incubated for 1–2 h at 25–35°C, thus enabling the repair of the cells. Double-strength violet red bile agar can then be overlaid on the plates and the plates incubated at 35°C for 24 h for selective growth of coliforms.[1–3]

D. Dilution Scheme, Plating, Incubation, Selection of Plates for Counting CFUs, and Reporting Results

The standard and recommended method books listed above have detailed descriptions on these steps. They should be followed as accurately as possible. The average of CFUs from duplicate or triplicate plates from a selected dilution should be used to report the counts of the specific microbial group per ml or g, or cm^2 of the food sample. This data should be used in the proper interpretation of microbiological quality of the food.

VI. QUALITATIVE METHODS TO ISOLATE MICROORGANISMS IN FOODS

A. Isolation of Pathogens

The main objective of this method is to determine whether a sample contains viable cells or spores of a specific pathogen. Foods are tested for several pathogens, such as *Salmonella*, *Esc. coli* O157:H7, *Lis. monocytogenes, Vibrio cholerae,* and *Shigella* spp., by the specific isolation procedure when necessary. For other pathogens, such as enteropathogenic *Esc. coli, Yer. enterocolitica*, and *Cam. jejuni*, isolation procedures are not generally used; instead, enumeration procedures are used.

An isolation procedure generally contains several steps, such as nonselective preenrichment, followed by selective enrichment, and then testing on an agar medium containing selective and differential agents. It is assumed that a food normally contains a low population of a pathogen as compared with associative microorganisms, and the pathogens could be in the injured state. The food sample (e.g., 25 g) is first preenriched in a nonselective broth and incubated for the injured cells to repair and then multiply in order to reach moderately high numbers (along with many other associated microorganisms). An aliquot is then transferred from the preenrichment broth to a selective enrichment broth and incubated. It is expected that during incubation, the specific pathogen and closely related microorganisms will selectively grow to a high number, whereas many of the associated microor-

ganisms will not grow. A small amount (≅0.01 ml) of the enrichment broth is then usually streaked on the surface of a prepoured selective-differential agar medium plate, which is then incubated for specified time for the colonies to develop. From the differential colony characteristics, the presence of a specific pathogen can be tentatively established.

This is generally considered a presumptive test. For confirmation, the cells from the characteristic colonies are purified and examined by the recommended methods for biochemical reaction profiles and serological reaction against a specific antibody. Isolation of a pathogen using the conventional methods can take 10 to 12 d, depending on a particular species.[1–3]

VII. TEST FOR BACTERIAL TOXINS IN FOODS

Sta. aureus and *Clo. botulinum* strains and several others (e.g., *Bac. cereus, Vib. parahaemolyticus*), while growing in foods, can produce toxins and cause intoxication or food poisoning among consumers. Specific methods have been developed to test the presence of toxins in the suspected foods.[1–3]

To detect *Sta. aureus* enterotoxin in foods, the recommended method is to initially extract the toxin from 100 g food and then to concentrate the toxin in 0.2 ml sterile water or 0.2 *M* saline. The presence of toxin in the concentrated extract is then assayed against specific antibody by the microslide precipitation method. This method is sensitive at 0.05 μg enterotoxin/ml of the extract. (Food poisoning can probably occur from consuming less than 1.0 μg of *Sta. aureus* enterotoxin A.) Methods such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and reverse passive latex agglutination assay (RPLA) can also be used in place of the microslide precipitation method.

To detect botulin, the toxin of *Clo. botulinum* strains, the recommended procedure is used to extract toxin from a food. To activate toxin of nonproteolytic types (B and E), a portion of the extract is then treated with trypsin. Both trypsinized and nontrypsinized (for proteolytic types A and B) extracts in 0.5-ml portions are then injected into mice intraperitoneally (two mice for each sample). Heated (100°C for 10 min) extracts are injected similarly (control). The mice are then observed up to 48 h for botulism symptoms and death. Typical botulism symptoms in mice, in sequence, are ruffling of fur, rapid and gasping breathing, weakness of limbs, and death due to respiratory failure.

Specific methods for testing toxins of some other pathogenic bacteria and molds have also been developed and listed in the recommended books as well as available from commercial producers.[1–3]

VIII. RAPID METHODS AND AUTOMATION

The traditional or conventional methods used for the quantitative or qualitative detection of microorganisms in foods and toxins take a relatively long time and are labor intensive. To overcome these difficulties, different rapid methods, many of

which are automated, have been developed to detect microbial loads, foodborne pathogens, and their toxins.[1,4,5] Some of these methods have been approved by regulatory agencies. In addition to being rapid, they are quite specific, sensitive, relatively accurate, and less labor intensive. However, their effectiveness in food systems with a low level of contamination needs further evaluation. Many new methods are constantly being developed, which differ mainly in technique. The principles, procedures, and applications in food of some of these methods are briefly presented. Exact methods and steps are provided by commercial firms.

A. Immunoassays for Rapid Detection of Pathogens

Several rapid and automated methods have been developed that rely on the specific antigen–antibody reaction and production of agglutination, color formation from chromogenic substrate, formation of an immunoband, or fluorescence.

1. Immunofluorescence Method

In this method, commercially available specific fluorescence-conjugated antibodies (against somatic or flagellar antigens of a pathogen) are mixed with an enriched medium suspected to contain the specific pathogen, such as *Salmonella* (antigen), on a glass slide. Following incubation and removal of reagents, the slide is examined under a fluorescence microscope for cells showing fluorescence on the cell wall or flagella, or both.

2. RPLA Method

This method has been developed to detect toxins of several foodborne pathogens, such as *Sta. aureus, Clo. perfringens, Bac. cereus, Vib. cholerae*, and enteropathogenic *Esc. coli*. The antibody of a specific toxin is immobilized on latex particles that are then mixed with a sample preparation suspected to contain toxin (antigen) in wells of microtitration plates. If the specific toxin is present in the sample, a diffused pattern will appear in the bottom; in its absence, a ring or button will appear.

3. Immunoimmobilization Method

This method was developed for motile pathogens such as *Salmonella* and *Esc. coli* O157:H7. The motile cells and antibody against flagellar antigen are applied on soft agar to enable them to diffuse in the same direction from opposite sides. When the bacterial cells and specific antibody meet, they form a visible arc due to the immobilization of cells.

4. Enzyme Immunoassay (EIA) or ELISA Methods

These methods were commercially developed and used to detect several foodborne pathogens and their toxins, namely, *Salmonella, Lis. monocytogenes,* and *Cam. jejuni*. The specific antibody is first allowed to bind on a solid surface (on a wall of

a microtitration plate) as per the directions of the commercial producer. Blocking agents (bovine serum albumin) are then added to block the additional protein-binding sites on the surface. The sample suspected of containing the antigen (pathogens or their toxins) is prepared as per requirement and then added to the well and incubated for the antibody–antigen reaction. After removing the unbound antigen, another antibody labeled with a specific enzyme (such as peroxidase) is added and incubated for its binding to the antigen to form a sandwich (antibody–antigen–antibody–enzyme). The unbound enzyme-linked antibody is then removed. The sandwich complex is finally detected by adding a chromogenic substrate specific for the enzyme (such as 4-chloro-1-naphthol for peroxidase), incubating for a specified time, and adding an enzyme inactivator to stop the reaction. The intensity of color development can then be measured to identify the presence of a specific pathogen or toxin. Instead of a chromogenic substrate, a specific fluorogenic substrate (such as 3-*p*-hydroxyphenyl propionic acid for peroxidase) can be used and the reaction can be measured with a fluorimeter. Automated systems for the ELISA test are available.

5. Magnetic Immunobeads Method

The objective of the method is to immunocapture the cells (antigen) of a specific pathogen present in a food suspension with the help of magnetic immunobeads. The immunobeads are plastic-covered ferrous beads coated with the specific antibody. When the beads are mixed with a sample, they capture the specific antigen (cells). The immunocaptured beads are then removed by a magnet and tested by specific methods for the presence of a target pathogen, such as *Lis. monocytogenes.*

B. Nucleic Acid Probe for Detection of Pathogens

1. Hybridization Method

In this method, a DNA probe consisting of a 20- to 4000-nucleotide base sequence unique to a group of similar pathogens, such as *Salmonella* spp., is prepared. The unique sequence can be identified in the DNA or rRNA in the cells or from the amino acid sequence of a toxin produced by the bacterial group. The DNA probe can then be obtained from the cell DNA or synthesized from the nucleotides. The probes are radiolabeled with ^{32}P if, after hybridization, autoradiography is used for their detection. In the nonisotopic colorimetric method, an enzyme (e.g., peroxidase) is bound to a specific protein (e.g., streptavidin), which in turn binds to specific ligands (e.g., biotin) on the DNA probe and can be used to produce a color reaction to aid in measurement.

Both isotopic and nonisotopic DNA probes specific for *Salmonella* and *Lis. monocytogenes* are commercially available and approved for use by regulatory agencies. DNA probes for toxigenic *Esc. coli* and *Yer. enterocolitica* are also available. The commercial companies provide step-by-step procedures for each technique. In general, there is an initial preenrichment step, followed by an enrichment step (which can be 6 h or more) to facilitate growth of target bacteria; lysis of cells;

hybridization of the DNA probe with cell DNA or rRNA; capture of the hybridized DNA containing the probe; and measurement of either radioactivity or color production, depending on the probe (i.e., if it is isotopic or nonisotopic). The methods are specific and relatively more rapid than traditional methods.

2. Polymerase Chain Reaction (PCR)

The PCR technique helps amplify a segment of DNA located between two DNA segments of known nucleotide sequence. It is thus possible to obtain large numbers of copies of a specific DNA segment from a small sample (50 ng), which in turn facilitates its detection by gel electrophoresis (nonisotopic) or by Southern hybridization (both isotopic and nonisotopic). The method is very sensitive and may not need preenrichment or enrichment steps. However, to eliminate confusion between dead and viable cells of a pathogen as the source of DNA, a short preenrichment step to increase the viable cell number of the target pathogen may be necessary. The method has been developed to identify such pathogens as *Lis. monocytogenes*, pathogenic *Esc. coli,* and *Vib. vulnificus*.

C. Measurement of Microbial Level

Several indirect methods to estimate microbial levels or loads in a food have been developed. They are relatively more rapid than the traditional CFU enumeration method and some are also automated.

1. Electrical Impedance Measurement

This method is based on the theory that as microorganisms grow in a liquid medium, they metabolize the uncharged or weakly charged substrate to produce highly charged small products (e.g., amino acids and lactic acid). This causes a change in the electrical impedance (resistance to flow of an alternating current) and conductance of the medium. By measuring these two parameters, bacterial growth in a medium as a function of time at a given temperature can be monitored. Impedance detection time (IDT) is the time required by microorganisms initially present in a food to reach $\geq 10^6$/ml. It is inversely proportional to the initial microbial load: a higher IDT means a lower initial load. From the IDT and the slope of the curve, the initial load as well as generation time of a microbial population can be calculated. Several automated systems are now commercially available that describe the technique as well as methods to interpret the data. By using selective conditions, the population of a specific bacterial group in a food can also be determined by this method.

2. Bioluminescence Methods

The bioluminescence method measures the ATP content in a sample as an indirect measurement of microbial load.[1,6] As only viable cells retain ATP, the amount of ATP is regarded as directly related to the microbial load in the sample. Using the

luciferin–luciferase (from firefly) system in the presence of Mg^{2+}, the ATP concentration in the lysed cells in a sample is measured. The method can detect as low as 10^{2-3} viable bacterial cells and ca. 10 yeast cells/g or /ml of a food. It can also be used to determine microbial population on equipment surfaces. The method is very rapid, and several automated systems with methods to use them are now commercially available.

In another method, the genes encoding bacterial bioluminescence (*lux* gene) from luminous bacteria (e.g., *Vibrio* spp.) have been cloned in some pathogenic, indicator, and spoilage bacteria important in food. Bacterial luciferase catalyzes oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde by molecular O_2 and emits light. Because only live cells can produce light, the bacterial strains containing the cloned *lux* gene can be used to determine the effectiveness of methods employed to kill cells or remove from food and food environments very rapidly. It is also used to detect injured bacteria incapable of producing light, but able to do so following repair of injury. Automated systems to measure light from the reaction are now commercially available.

Many other rapid methods, automated systems, and miniature versions of traditional methods are now commercially available. Some have been discussed in more detail in the references listed and can be consulted.

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